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(54) Title: PURIFIED RAP GAPs, RAP GAP SEQUENCES, AND USES THEREOF

(57) Abstract

This invention presents substantially purified rap GAPs (ras proximate guanine triphosphatase activating proteins); and methods for purifying the proteins using either successive chromatographic steps or monoclonal antibodies to the proteins or to the partial amino acid sequences of the protein. The DNA and predicted amino acid sequences of a rap GAP are presented, as well as antibodies against rap GAP and methods for producing such antibodies. Also presented are methods for identifying anticancer therapeutics using these rap GAPs.

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PURIFIED RAP GAPs, RAP GAP SEQUENCES, AND USES THEREOF

This invention relates generally to the field of molecular biology, and specifically to rap GAPs (ras proximate guanine triphosphatase activating proteins); and methods and material(s) for identifying anti-cancer therapeutics using these molecules.

Rap is a protein capable of reverting ras oncogene transformed cells to a normal cell phenotype. Rap GAP is a protein capable of activating the intrinsic GTPase activity of rap. The following presents a background on the ras, rap proteins and their respective GAPs.

Ras:

Ras proto-oncogenes (for example, H-ras, K-ras, and N-ras) encode an evolutionary conserved family of proteins, ras p21 proteins, widely present in eukaryotic cells. Mutant and viral forms of ras p21 have been implicated in many cancers. The ras oncogenes have been implicated in the development of a variety of tumors, and have been shown to be involved in about 10 to 40% of the most common forms of human cancer. See Varmus, H., 1984, Ann. Rev. Genetics, 18:553 and Barbacid, M., 1986, in Important Advances in Oncology, DeVita, D., (ed.), pgs 3-22, Philadelphia: Lippincott. For example, ras oncogenes have been consistently identified in carcinomas of the bladder, colon, kidney, liver, lung, ovary, pancreas and stomach. They have also been identified in hematopoietic tumors of lymphoid and myeloid lineage, as well as in tumors of mesenchymal origin. Futhermore, melanomas, teratocarcinomas, neuroblastomas, and gliomas have been shown to possess ras oncogenes.

While oncogenic ras p21 is an effective tumorigenic agent, normal ras p21 can induce the malignant phenotype. Chang, et al., 1982, Nature, 297:7479 and Pulciani, et al., 1985, Mol. Cell Biology, 5:2836. Further, amplification of normal ras gene has been observed in several human tumors, and has an apparent incidence of about 1%. Pulciani, Id., Yokota, et al., 1986, Science, 231:261.

Ras p21 (hereinafter also referred to as ras) exhibits both GTP and GDP binding, and GTPase activities. Barbacid, M., 1987, Annal Rev. Biochem., 56:779-827; Takai, Y., et al., 1988, Progress in Endocrinology, Imura, H. et al. (eds.) 2:995-1000; Elsevier Science Publishers, B.V., Amsterdam). It binds guanine nucleotides and converts bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP) by an intrinsic guanosine triphosphatase (GTPase) activity. Trahey, M., et al., 1987, Science, 238:542-545. Cytosolic GAP (GTPase Activating Protein) has been found to

stimulate the GTPase activity of normal ras p21, but not the GTPase activity associated with the oncogenic mutants. Id. Both the normal ras p21 and oncogenic ras p21 proteins, regardless of their phylogenetic origin bind GTP and GDP, and possess intrinsic GTPase activity. Teneles et al., 1985, Nature, 313:700. The significance of these biochemical properties has been demonstrated as follows.

First, microinjection of anti-ras p21 antibodies that interfere with guanine nucleotide binding reverses the malignant phenotype of NIH 3T3 cells transformed by ras oncogenes. See Clark et al., 1985, PNAS (USA), 82:5280 and Feramisco, et al., 1985, Nature, 314:639.

Second, ras oncogenic proteins that exhibit mutations which result in the inability of p21 to bind guanine nucleotides do not transform NIH 3T3 cells. Willunser, et al., 1986, Mol. Cell Biology, 6:2646.

Third, some ras oncogenes produce p21 proteins that have much reduced GTPase activity compared to their normal cellular counterparts.

Rap:

There are at least 25 distinct low molecular mass GTP-binding proteins represented in eukaryotic cells. These proteins exhibit homology to ras p21, particularly in regions known to be involved in the binding and hydrolysis of GTP. Examples of these proteins are rap, rho, and G25K. Rap is an acronym for ras-proximate which denotes that it is a ras related GTP binding protein. Rap has been purified. Kawata, M., et al., 1988, J. Biol. Chem., 263: 1965. Recently, two human cDNAs encoding raps have been isolated and cloned. Pizon, V., et al., 1988, Oncogene, 3:201-204. It was found that the rap and ras proteins share many structural properties, for example, rap proteins share amino acid sequence similarities with regions 10 to 17 and 57 to 63 of the ras protein. Id. In fact, the effector regions of ras and rap, regions 32 to 40, are identical. Transfection of ras-transformed cells with human cDNA encoding rap results in the phenotypic reversion of the transformed cells. Kitayama, H., et al., 1989, Cell, 56: 77-84. The suppression of ras-transformed phenotype by the cDNA encoding rap operates in a mRNA level dependent manner. Id.

Based on similarities in the sequences of rap and ras, Kitayama et al. hypothezised that rap might antagonize the activity of ras proteins by competing with ras protein for common target or regulatory protein. Alternatively, since ras was believed to be involved in the transduction of growth promoting signal in fibroblasts, Kitayama et al. hypothesized that rap might serve as a G-protein involved in the transduction of growth inhibitory signal.

Additionally, there are GTPase activating proteins associated with rap (the rap GTPase activating protein is hereinafter referred to as rap GAP) which may play a role in the regulation of the ras oncogene product(s). Rap GAP activity has been identified in the cytosols of bovine brain and human platelets. Two forms of cystosolic rap GAPs obtained from bovine brain were reported by Kikuchi, A., et al., 1989, J. Bio. Chem., 263:9133-9136. According to Kikuchi, et al., these rap GAPs stimulated the GTPase activity of rap but not that of c-Ha-ras p21 (another name for rap p21), rho, and smg-25A (smg-25A is also known as rab3). These rap GAPs did not stimulate the dissociation of guanosine 5'-3-O-(thio)triphosphate and GDP from rap. Further, these rap GAPs did not themselves have GTP/GDP binding activity nor GTPase activity. The M_r of the two rap GAPs are believed to be: 300-400 x 10^3 and 90-100 x 10^3; and 250-330 x 10^3 and 80-90 x 10^3 respectively based on gel filtration chromatography and sucrose density gradient ultracentrifugation, respectively. However, it should be noted that these rap-GAPs were not purified to homogeneity. In another paper, Kikuchi and colleagues presented two rap GAPs partially purified from cytosol of human platelets. Ueda, T., et al., 1989, Biochem. & Biophy. Res. Comm., 159(3):1411-1419. However, these rap GAPs are estimated to have M_r of 2.5 - 3.5 x 10^5.

One aspect of the invention presents substantially purified rap GAP with a molecular weight of about 88,000. In particular, two rap GAPs are disclosed: rap GAPm and rap GAPb.

Another aspect of the invention presents the DNA and predicted amino acid sequences of rap GAP.

Another aspect of the invention presents antibodies to rap GAP, synthetic peptides for producing the antibodies, and methods for producing the antibodies.

Another aspect of the invention presents peptides comprising partial amino acid sequences of rap GAP.

Another aspect of the invention presents methods for purifying rap GAP using successive chromatographic steps.

Another aspect of the invention presents methods for isolating membrane associated rap GAP from tissue and cell sources.

Another aspect of the invention presents methods for purifying rap GAP using monoclonal antibodies to rap GAP or to peptides comprising partial amino acid sequences of rap GAP.

A further aspect of the invention presents methods for identifying anti-cancer therapeutics using material(s) and methods consisting of rap GAP.

Figure 1 presents an analysis of rap protein purified from human platelet membranes.

A) SDS-PAGE gels were either stained for protein with Coomassie Blue (Coo Blue) or subjected to electroblotting. The blots were incubated with antibodies reactive to rap, G25K, rac-1, or p21 as indicated below the panels. 100 ng of each of the purified proteins or 50 µg of plasma membrane (PM) for HL60 cells were applied to the gel lanes.

B) Comparison of the amino terminal amino acid sequence obtained with the purified protein and the sequence deduced from the rap1b cDNA.

Figure 2 presents the association of rap GAP with the plasma membranes from differentiated HL60 cells. The results are expressed as a percentage of the formylpeptide binding or rap GAP activity determined for membranes incubated in buffer only.

Figure 3 presents the detergent solubilization of rap GAP activity.

A) Time course: The Rap GAP assays were carried out as described below, except that a 10-fold volume of all components was used. At the indicated times 20 µl aliquots were removed, diluted and then filtered through nitrocellulose. Percent remaining indicates nonfilterable [γ-^{32}P]GTP relative to buffer control at zero time. Extraction buffer (closed symbols); 5 µl of membrane extract (open symbols).

B) Dilution series: The indicated volumes of extract were diluted to 2 µl and assayed for rap GAP activity. Percent hydrolysis is the loss of nonfilterable [γ^{32}P]GTP relative to buffer control.

Figure 4 presents the elution profiles of rap GAP activities of cytosolic and solubilized membrane rap GAP on size exclusion HPLC.

A) Chromatogram: 500 µl (4 mg/ml) of HL60 cell cytosol (open symbols) or 500 µl (3.1 mg/ml) of membrane extract (closed symbols) were applied to an SEC-250 HPLC column. Fractions (0.5 ml each) were assayed for rap GAP activity. Elution positions of the standard proteins are indicated by the arrows. Vi and Vo designate internal and excluded volumes, respectively.

B) Estimation of molecular size: Kav = Ve-Vo/Vi-Vo, where Ve is the elution volume of the protein. Molecular size values used for the standard proteins were carbonic anhydrase, 23 Å; ovalbumin, 30.5 Å; IgG, 48 Å; and catalase, 52 Å.

Figure 5 presents S-Sepharose chromatography of membrane extracts.

A) Chromatogram: rap GAP activity ( ); Absorbance at 280 nM (solid line); NaCl concentration (stippled line). (2 µl of each fractions was assayed for rap GAP activity)

B) SDS-PAGE: 30 µl of each of the indicated fractions or column load(L) was applied to the gel. The gel was stained with Coomassie blue. Values at left indicate molecular weights (x 10^{-3}) of standard proteins.

Figure 6 presents a Mono Q HR5/5 FPLC column (5 x 50 mm) chromatography.

A) Chromatogram: Symbols are as described in the legend to Figure 5. 2 µl of each fraction was assayed for rap GAP activity.

B) SDS-PAGE: 10 µl of the column load (L) or 30 µl of each of the indicated fractions was applied to the gel. The gel was stained for protein with silver. Mobilities of standard proteins indicated at left were determined on a separate gel by co-electrophoresis with the column load.

Figure 7 presents Hydroxylapatite HPLC.

A) Chromatogram: rap GAP activity ( ); Absorbance at 280 mM (solid line); Concentration of potassium phosphate (stippled line), 2 µl of each fraction was assayed for rap GAP activity.

B) SDS-PAGE: 15 µl of the column load(L) or 30 µl of each of the indicated fractions was applied to the gel. The gel was stained for protein with silver. Molecular weights (x 10^{-3}) of standard proteins (STD) are at left.

Figure 8 presents SEC-250 HPLC.

A) Chromatogram: 2 µl of each fraction were assayed for rap GAP activity as in the preferred assay method, except that the incubation period was extended to 20 minutes.

B) SDS-PAGE: analysis of the SEC-250 HPLC elution profile. 40 µl of each of the indicated fractions or the column load (L) was applied to the gel. The gel was stained using silver. The molecular weight (x 10^{-3}) of the standard proteins are indicated.

Figure 9 presents cation exchange SP-5-PW HPLC of rap GAP purified from bovine brain membranes.

A) Chromatogram symbols are as defined in Figure 5: 2 µl of a 1/75 dilution of each fraction was used in the rap GAP assay.

B) SDS-PAGE: 15 µl of each of the indicated fractions or column load(L) was applied to the gel. The gel was stained with Coomassie blue. Molecular weights (x 10^{-3}) of the standard proteins are indicated at left.

Figure 10 presents the DNA and the predicted amino acid sequences of HP3-12 and HUB10A; the amino acid sequences of the peptide for raising polyclonal rabbit antibodies against rap GAP (denoted "S" in the drawing) is also shown.

Figure 11 presents:

A) S-Sepharose chromatogram of AcRG4 infected baculovirus cell lysate. Rap GAP activity ( ); NaCl concentration ( ). 2 µl of a 1/1,000 dilution of each fraction was assayed for rap GAP activity.

B) SDS-PAGE analysis of the S-Sepharose chromatography elution profile, 5 µl each of the indicated fractions or column load (L) or flow through (FT) was applied to the gel. The gel was stained with Coomassie blue. Molecular weights (10^{-3}) of the standard proteins are indicated at left.

Figure 12 presents Western blot of rap GAP purified from different sources. Lane M contained 15 µl of rap GAP lysate from the membrane fraction of Spodoptera frugiperda (Sf9) cells infected with AcRG9; Lane C contained 0.3 µl of rap GAP lysate from the cytosol fraction of Sf9 cells infected with AcRG9; the membrane fractions were resuspended in the same volume as that of the cytosolic fraction. Lane "Bov Br" contained 15 µl of a 0.01 mg/ml solution of rap GAP purified from bovine brain membrane. The rabbit antibodies used were raised against the synthetic peptide based on a partial rap GAP sequence (indicated by "S" in Figure 10). The antibodies were affinity purified against the synthetic peptide. The antibody production and purification was conducted according to the procedure outlined in Example 1.

The invention described herein draws on both published and unpublished work. By way of example, such work consists of scientific papers, pending patent applications, and patents. All of this work, cited previously or below are hereby incorporated by reference.

The present invention presents substantially purified rap GAPs. In particular, it provides descriptions of two purified rap GAPs. Rap GAPb was purified from bovine brain membranes, and rap GAPm was purified from plasma membrane of differentiated HL60 cells. The purification methods, applicable to both native and recombinant rap GAPs which include for example, membrane and cytosolic rap GAPs, are also presented. The characterization of the two rap GAPs is also described. The invention further presents the partial amino acid sequence of rap GAP, specifically that of rap GAPb. Peptides comprising the partial amino acid sequences of rap GAPb are also presented. Antibodies directed against these rap GAPs or peptides comprising the partial amino acid sequences of rap GAP are also described. Finally, methods for purifying rap GAP using these antibodies is also presented. These rap GAPs, and the fragments derived therefrom are particular useful as therapeutics for cancer.

Since the purified rap GAPs are obtained from crude preparations, the order of discussion of the invention will be: purification, characterization, and sequencing methods for rap GAPm and rap GAPb, respectively; the partial amino acid sequence of

rap GAPb; synthesis of antibodies to the rap GAPs; peptides comprising the partial amino acid sequences of rap GAP; purification of rap GAP using monoclonal antibodies to rap GAP or to peptides comprising partial amino acid sequences of rap GAP; the therapeutic uses of rap GAP and their fragments.

It will be understood by those skilled in the art that rap GAP may exist as aggregates or multimers under certain conditions, and that these forms are intended to come within the scope of the definition. Moreover, the definition is intended to cover fragments of rap GAP that exhibit activity.

It will be appreciated that the precise chemical structure of the native and recombinant rap GAPs, and their fragments may depend on a number of factors. As the proteins contain ionizable amino and carboxyl groups, it is apparent that they may be obtained in acid or basic salt forms, or in neutral forms. It is further apparent that the primary amino acid sequences may be augmented by derivatization using sugar molecules (glycosylation) or by other chemical derivatizations involving covalent or ionic attachment to the proteins with, for example, lipids, phosphate, acetyl groups and the like, often occurring through association with saccharides. These modifications may occur in vitro or in vivo, the latter being performed by a host cell through post-translational processing systems. It will be understood that such modifications, regardless of how they occur, are intended to come within the definition of the proteins so long as the activities of the proteins, as defined herein, are not significantly altered.

As used herein, "rap GAPb" is defined as rap GAP which is obtained from bovine brain, and is membrane associated.

As used herein, "rap GAPm" is defined as rap GAP which is obtained from differentiated HL60 cells, and is membrane associated.

As used herein, "rap·GTP" is defined as the GTP-bound form of rap.

As used herein, "rap·GDP" is defined as the GDP-bound form of rap.

As used herein, "rap GAP-rap·GTP" is defined as the complex comprising rap GAP bound to rap·GTP.

As used herein, the preface "pAcRG" denotes a plasmid containing a particular DNA insert; whereas the preface "AcRG" denotes the virus containing the particular DNA insert.

As used herein, "chromatography" is defined to include application of a solution containing a mixture of compounds to an adsorbent, or other support material which is eluted, usually with a gradient or other sequential eluant. Material eluted from the support matrix is designated eluate. The sequential elution is most routinely performed by isolating the support matrix in a column and passing the eluting solution(s), which changes affinity for the support matrix, either stepwise or preferably by a gradient,

through the matrix. It will be appreciated that encompassed within the definition "chromatography" is the positioning of the support matrix in a filter and the sequential administering of eluant through the filter, or in a batch-mode.

Although any similar or equivalent methods and materials may be employed in the practice or testing of the present invention, the preferred methods and materials are now described.

Rap GAP activities are found in cell membrane and cytosol. Thus, a general scheme for rap GAP isolation consists of isolating the molecule from the cytoplasm or membrane of appropriate cells, tissues or organs. More specifically, rap GAP is prepared by isolating the molecule from the cytosol or membrane using any number of techniques including freeze thawing, sonication, mild detergent extraction, and nitrogen cavitation, etc. This procedure is preferably carried out in a physiologically buffered solution containing one or more protease inhibitors. Moreover, to further inhibit protease activity, especially those proteases that rely on metal ions for activity, the extraction solution may contain metal ion chelators. The preferred extraction solution is a physiologically balanced salt solution containing the chelator ethylenediaminetrichloroacetic acid (EDTA), plus the protease inhibitor phenylmethylsulfonylfluoride (PMSF). The metal ion chelator(s), as well as the protease inhibitor(s) are present at concentrations that effectively inhibit proteolysis, preferably about 1 mM, and 0.2 to 0.5 mM, respectively. However, it will, of course, be appreciated by those skilled in the art that since the types and amounts of proteases vary depending on the starting material used to extract rap GAP, the concentrations that the protease inhibitors or chelators are used at, if indeed used at all, will also vary.

In the case of purification of cytosolic rap GAP, the mixture containing rap GAP is clarified by centrifugation, or in other ways to remove insoluble material from the aqueous fraction. If the aqueous fraction contains low amounts of rap GAP it can be concentrated by any one of several techniques well known to those skilled in the art, including high salt precipitation, for example, with ammonium sulfate, or by ultrafiltration. If rap GAP is concentrated by precipitation, it is preferably subsequently resuspended in a suitable physiologically balanced salt solution containing protease inhibitor(s).

In the case of isolation of the membrane rap GAP, variations of the methods described above are used. The general method for isolating and purifying membrane proteins is presented by Van Renswoude, et al., 1984, Methods in Enzymology, 104:329. Briefly, the first step in isolating the membrane rap GAP constitutes fractionating the source tissues or cells to obtain highly purified plasma membranes. To minimize the risk of degradation by endogenous or exogenous proteolytic activity,

this step is carried out quickly in the cold and with the addition of protease inhibitors, such as PMSF, leupeptin, and N-tosyl-L-phenylalanylchloromethyl ketone (TCPK). After the desired membrane fraction has been obtained, soluble proteins that are still associated with the membrane and peripheral membrane proteins may be removed by one or more of the following techniques, including exposing the membrane fraction to the following chemicals: KCl or NaCl in relatively high concentrations of between 0.15 to 3.0 M; washing with buffers of acid or basic pH, or with sodium carbonate; chelating agents such as EDTA or EGTA up to 10 mM; chaotropic ions (I^-, Br^-, ClO_4^-, SCN^-) at high concentrations of between 2 to 4 M; phenolic compounds such as lithium 3, 5-diiodosolicylate; protein-modifying reagents such as p-chloromercuribenzoate, p-chloromercuribenzene sulfonate, and acid anhydries such as succinic or maeic anhydrides; reducing agents such as 2-mercapthoethanol or dithiothreitol (DTT). However, contrary to the suggestion in the Van Renswoude article, Id., in the case of rap GAP purification, denaturing agents are not recommended as they destroy the activity of rap GAP (see Figure 2).

After an appropriate interval for the reagents to take effect, the mixture is then centrifuged to recover the membranes. Next, the membranes are solubilized with an appropriate detergent to liberate its constituents, lipids, and membrane proteins. The following detergents may be used: 1) nonionic detergents such as octylglucoside, Nonidet P-40 (hereinafter referred to as NP-40 and commercially available from Sigma Chem. Co., St. Louis, MO); 2) zwitterionic detergents, such as 3- (3'-cholamidopropyl) dimethylammonia-1-propanesulfonate (CHAPS); 3) ionic detergents, such as acetylammonium bromide and sodium dodecyl sulfate (SDS); and 4) bile salts, such as deoxycholate.

Alternatively, instead of detergents, the membrane protein can be extracted with organic solvents. Examples of the organic solvents that can be used are n-butanol, n-pentanol, aqueous phenol, pyridine, chloroform methanol mixture, acetic and formic acid, 2 chloroethanol, etc.

Depending on how the mixture of the membrane proteins is solubilized, the following are possible choices of fractionating methods: phase separation, gel filtration, ion-exchange chromatography, affinity chromatography, covalent chromatography, hydrophobic interaction chromatography and HPLC, etc. Throughout the above, the isolation and purification steps can be monitored by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), and assay for rap GAP activity, both are described infra.

In the preferred embodiment for isolating membrane rap GAP, the source tissue or cells are fractionated, preferably the cells are lysed by nitrogen cavitation. The

membrane rap GAP is isolated in the cold, quickly, and in the presence of PMSF and leupeptin. The soluble proteins and peripheral membrane proteins are then removed by a combination of washing with NaCl; chelating agents such as EDTA and reducing agents such as DTT. After an appropriate incubation period, the mixture is centrifuged to recover the membrane fraction. The membrane fraction is then solubilized with detergents such as the non-ionic detergent NP-40, and bile salts such as deoxycholate. The preferred concentration range of NP-40, deoxycholate, DTT, and PMSF, are preferably 0.5 to 1.0%; 0.5 to 1.0%; 0.5 to 1 mM; and 0.2 to 0.5 mM, respectively. The EDTA concentration is preferably about 1 mM.

Detergent solubilized rap GAP, either the cytosolic or membrane forms, may be purified as follows. In the preferred embodiment, Method 1 (for example, as used in the purification of rap GAP from bovine brain membrane), insoluble material is first removed and the soluble rap GAP fraction subjected to cation exchange chromatography, a sizing column, and an anion exchange chromatography. The order of the steps can be varied.

The alternative preferred purification procedure, Method 2, (for example as employed in the purification of rap GAP from differentiated HL60 cells), is as follows. The soluble rap GAP is subjected to cation and anion exchange chromatography, and hydroxylapatite HPLC (High Pressure Liquid Chromatography) (commercially available from Bio-Rad Laboratories, Richmond CA). Again, the order of the steps can be varied. In both methods, the protein purification procedures were carried out at 4°C.

The following discusses Methods 1 and 2 in more detail.

Method 1:

After detergent solubilization, described supra, a buffer solution is added to the protein mixture containing rap GAP. The solution is preferably phosphate buffered, but other buffers, for example, Tris and MES can also be used. An ion exchange chromatographic step compatible with the buffer is then used to purify the protein mixture. In Method 1, the solution containing rap GAP is purified by cation exchange chromatography. Next, the eluate of the cation exchange chromatography is passed through a sizing column appropriate for excluding proteins of molecular size different from rap GAP. Then, the fractions exhibiting rap GAP activity are subjected to an anion exchange chromatography followed by a cation exchange chromatography.

Thus, the preferred purification scheme will consist of applying rap GAP in a phosphate buffered solution to a cation exchange chromatography, and eluting rap GAP therefrom, preferably using solutions which alter the pH or conductivity of the solution. More preferably, rap GAP will be eluted by applying either a gradient or non-

gradient salt solution, and most preferably will be eluted using a linear gradient of sodium chloride over the range of about 0-0.5 molar.

The preferred cation exchanger is a S-Sepharose cation exchanger (commercially available from Pharmacia/LKB Upsula Sweden). The S-Sepharose cation exchanger is an elastic 3-dimensional network composed of cellulosic backbones cross-linked with vinyl polymer containing pendant sulfopropyl functional groups. The matrix is preferably adapted for radial flow passage of the rap GAP solution. The flow rate of the solution through the matrix will depend upon the size and geometry of the matrix used. It will be apparent to those skilled in the art, however, that care should be taken to avoid exceeding the unit capacity of the matrix with rap GAP. If the capacity is exceeded, rap GAP will not be totally retained and excess unretained rap GAP will be present in the effluent. The capacity of the matrix to retain rap GAP can be monitored by assaying for rap GAP in the effluent using one of the assays described below. The eluant is then passed through a size exclusion column appropriate to exclude proteins of molecular size different from rap GAP. The preferred size exclusion column is Sephacryl S-200 to S-300 (commercially available from Pharmacia/LKB, Upsula, Sweden), the latter is more preferred.

Fractions containing rap GAP are then prepared for the third chromatographic step, the anion exchange chromatography. This consists of combining the fractions and adjusting the solution to a pH, and ionic strength compatible with anion exchange chromatography. A variety of anion exchangers are available, and depending on the type employed, the concentrations of these reagents will vary. The general procedures for preparing and using these matrices are known to those skilled in the art. The preferred anion exchanger is Mono Q HR 5/5 FPLC (commercially available from Pharmacia/LKB, Upsula, Sweden). It is prepared by equilibrating it with a solution containing Tris buffer at a pH of 8.5. More preferably, the solution will consist of Tris, pH 8.5 plus a reducing agent, a metal chelator, and a protease inhibitor. The concentrations of the metal chelator and protease inhibitor will vary depending on how extensively rap GAP is proteolysed, and whether the proteases responsible are activated by metal ions.

The solution is then passed through the anion exchange matrix whereupon rap GAP binds to the matrix. Rap GAP is subsequently eluted from the matrix using solutions which alter the pH or conductivity. The preferred elution method consists of eluting rap GAP using a linear salt gradient ranging from 0-0.5 molar sodium chloride. The eluant is further dialysed against cation exchange buffer and passed through a cation exchanger. The preferred cation exchange buffer is S-Sepharose (described in Example 1) buffer at pH 6.5 and the cation exchanger is PW-5 HPLC column

(commercially available from Bio-Rad Laboratories, Richmond, California) and the column is eluted with 0-0.5 M NaCl. It should be noted that the above purification steps need not be conducted in the order presented but may be conducted in any order that allows purification to be effectuated.

Method 2:

Similar to Method 1, the step after detergent solubilization in Method 2 also involves the addition of a buffer as described in Method 1, to the protein mixture containing rap GAP. Similarly, a cation exchange chromatography is used. This is followed by an anion exchange chromatography and a hydroxylapatite HPLC (commercially available from Bio-Rad Laboratories, Richmond, California). The preferred cation and anion exchange chromatography is S-Sepharose cation exchanger and Mono Q HR5/5 FPLC, respectively. The general discussion regarding these cations and anion exchange chromatography is similar to Method 1.

Finally, it should be noted that while the preferred applications of the ion exchange materials described herein are in a column format, it will be appreciated that they may also be used in batch format as well.

Because rap GAP is a protease-sensitive molecule that is broken down into lower molecular weight species having rap GAP activity, in a preferred embodiment of the invention the entire isolation and purification procedure is carried out rapidly in the cold to reduce protease activity. In general, this temperature is in a range below 10°C, with a preferred temperature range being about 2-8°C. Most preferred is a temperature of about 4°C.

The purity and activity of rap GAP obtained by the above purification methods can be monitored by the rap GTPase assay described below, and by electrophoretic procedures using sodium dodecyl sulfate polyacrylamide gel electrophoresis (hereinafter referred to as SDS-PAGE). SDS-PAGE was performed essentially as described in Laemmli, U.K., 1970, Nature, 227:680-685. Gels were stained for protein using silver as described in Guilian, G.G., et al., 1982, Anal. Biochem., 129:277-287, or with Coomassie blue.

A preferred embodiment purification scheme consists of isolating rap GAP from the membranes of HL60 and bovine brain cells, described in the Examples below.

The starting material for a rap GAP assay is rap protein. The rap protein can be obtained from several sources, using methods known in the art, e.g., as described in Kawata, M., 1988, J. Bio. Chem., 263:18965. The rap protein as used in the assay for the GTPase activating protein herein was purified from human platelet cells.

The purification method was essentially as described for the purification of rac and G25K (Polakis, P., et al., 1989, J. Biol. Chem., 264:16383-16389). The purification procedure was carried out at 4°C. Briefly, a particulate preparation of human platelets was extracted with 1% sodium cholate. The extract was chromatographed through DEAE-Sephacel and the GTP-binding activities were assayed as described in Polakis, P., Id. A peak of GTP-binding activity eluting at a lower salt concentration than the major peak was pooled and concentrated. This activity was then chromatographed sequentially through Ultrogel AcA-34, heptylamine-Sepharose, hydroxylapatite and finally DEAE-Fractogel. The resulting preparation is shown in the SDS-PAGE and immunoblots of Figure 1. The electroblotting was carried out at 0.3 Amps for 90 minutes in Tris/glycine (25/192 mM) containing 20% methanol. The blots were blocked with a 1% powdered milk solution and reacted overnight with the primary antibodies in Tris buffered saline containing 0.05% Tween 20. Anti-mouse and anti-rabbit IgG horse radish peroxidase conjugated secondary antibodies (commercially available from Bio-Rad Laboratories, Richmond, CA) were used at dilutions of 1/10,000 and 1/20,000, respectively. The blots were developed using the ECL detection system (Amersham, UK). The gels were either stained for protein with Coomassie blue (Cooblue) or subjected to electroblotting. The blots were incubated with antibodies reactive to rap, rac-1, G25K, or ras p21. These antibodies are specifically reactive to low molecular mass GTP binding proteins. These antibodies were raised against synthetic peptides corresponding to the following sequences: rap-1A, DLVRQINRKTPVEKK; rac-1 (described in Polakis, P., et al., 1989, J. Biol. Chem., 264:16383-16389), CPPPVKKKRKRK; and G25K (described in Polakis, P., et al., 1989, Biochem. Biophys. Res. Commun., 160:25-32), NVFDEAILAALEPPEPK. These antibodies were affinity purified against their respective immobilized peptides essentially as described in Mumby, S., et al., 1988, J. Biol. Chem., 263:2020-2026. The anti-ras p21 antibody is a mouse monoclonal IgG raised against a synthetic peptide corresponding to the sequence within residues 29-44 contained in ras p21. This antibody has been previously characterized and designated 6B7 in Wong, G., et al., 1986, Cancer Res., 46:6029. 100 ng of each of the purified protein or 50 µg of plasma membrane (PM) from HL60 cells were applied to the gel lanes. The amino terminal sequence (Figure 1B) was determined from material blotted onto polyvinylidene difluoride using methods described by Matsudaira, P., 1987, in J. Biol. Chem., 262:10035-38.

The rap protein used in these studies was purified to homogeneity from extracts of human platelet membranes. A reduced SDS-PAGE stained for protein shows a single polypeptide with a molecular mass of approximately 24 kD that comigrated with

the GTP binding activity during purification (Figure 1A). Amino-terminal sequence analysis of this protein revealed a 100% homology to the NH_2-terminal sequences predicted from the rap 1 cDNA (Figure 1B). An IgG directed against the rap-1A carboxy terminal sequence, DVLRQINRKTPVEKK, recognized the purified protein, as well as a 24 kD polypeptide present in HL60 cell plasma membranes (Figure 1A). The purified protein was also recognized by an antibody specifically reactive to the effector site sequence of p21 ras, which is also contained in rap (Figure 1A). Also tested were antibodies specifically reactive to two other ras-related proteins found in platelets, G25K and rac1 (Polakis, P. et al., 1989, Biochem. Biophys. Res. Commun., 160:25-32; and Polakis, J. Biol. Chem., 264, supra). Neither of these IgGs reacted with the purified rap preparation (Figure 1A).

Rap GAP may be assayed in vitro, and several different types of in vitro assays can be performed. For example, an assay may involve measuring the presence of GDP resulting from the hydrolysis of GTP. This assay involves combining in an appropriate physiologically buffered aqueous solution, empirically determined optimal amounts of normal cellular rap, and [α-^{32}P]GTP, plus rap GAP. The solution may also contain protease inhibitors and a reducing agent. Also, since cations greatly stimulate rap GAP activity they should be present in an effective amount. The preferred cation is magnesium chloride.

The reaction solution is incubated for various times and may be conducted at temperatures typically employed to perform enzymatic assays, preferably 10-40°C, and more preferably at 23°C. At the appropriate times aliquots are removed and assayed for [α-^{32}P]GDP. This is readily accomplished by first separating rap containing bound [α-^{32}P]GDP from the other reactants in the solution, particularly free [α-^{32}P]GTP. This can be achieved by immunoprecipitating rap with antibodies which will precipitate rap. Immune precipitation techniques and antibodies which will precipitate rap are known, and routinely employed by those skilled in the art. [α-^{32}P]GDP, is released from the immune precipitate preferably by dissolving the sample in a denaturing detergent at an elevated temperature, more preferably in 1% sodium dodecyl sulfate at 65°C for 5 minutes, and chromatographing the mixture on a suitable thin layer chromatographic plate. The chromatography is preferably carried out on a PEI cellulose plate in 1 M LiCl. [α-^{32}P]GDP is identified by its mobility relative to a known standard using suitable radiodetection techniques, preferably autoradiography.

An alternative assay for rap GAP activity is to substitute gamma labelled ^{32}P-GTP for α-labelled ^{32}P-GTP in the above assay system, and assay for free ^{32}P labelled phosphate using activated charcoal. This assay can be carried out as described by Tjian et al., 1980, Cold Spring Harbor Symp. Quant. Biol., 44:103.

An additional assay does not involve immune precipitation. Rather, an aliquot from a rap GAP assay reaction mixture described above can be directly subjected to PEI cellulose chromatography in 1 M LiCl. This assay, however, is most useful for assaying solutions having substantially purified rap GAP.

The preferred rap GAP assay as used in the Examples described below is as follows, unless specifically modified in the Examples.

The rap-[γ-^{32}P]GTP complex was formed by adding 10 µl of purified rap (50 µg/ml in 20 mM Tris, pH 8, 0.1 M NaCl and 0.1% Lubrol) to 20 µl of 20 mM Tris, pH 7.5, containing 30 µM $MgCl_2$ and 0.75 µM [γ-^{32}P]GTP (1500 Ci/mmol) (from ICN, Irvine, California) and incubating the mixture at 30°C for 10 minutes. Two µl of the rap GAP sample or buffer control was added to 16 µl of 25 mM Tris, pH 7.5, containing 6.25 mM $MgCl_2$, 1 mM dithiothreitol, 625 µM GTP, 1.25 mg/ml bovine serum albumin, 0.06% deoxycholate and 0.06% NP-40. Following the addition of 2 µl of the rap [γ^{32}P]GTP complex, the samples were incubated at 23°C for 10 minutes or for longer periods of time where noted. Reactions were stopped by adding 4 ml of ice cold 25 mM Tris, pH 7.5, containing 0.1 M NaCl and 5 mM $MgCl_2$ and then immediately filtered through nitrocellulose by rapid vacuum filtration. Filters were washed 3 times with 4 ml of the same buffer, dried and the radioactivity quantitated by liquid scintillation counting. Typically, 50,000-200,000 CPM remained associated with rap in the absence of rap GAP activity. The activities are expressed as the percentage of [γ-^{32}P]GTP bound to rap that was hydrolyzed relative to the buffer control.

The rap GAP protein, or fragments derived therefrom can be sequenced using standard techniques known to those skilled in the art. In the event that rap GAP is isolated having a blocked amino terminal end, internal sequencing can be achieved by fragmenting the molecule such as, for example, with lysyl endopeptidase, and sequencing one or more of the resulting fragments. Rap GAPb has the following partial amino acid sequences: F(G)VSTKLPFT(W)DXA(Q)QL and FLKKAKA (wherein X denotes unknown residue and parenthesis indicates uncertainty in the residue within the parenthesis).

Further experiments were conducted to obtain additional purified rap GAP_b and the following partial sequences: IASNFL(P)AYIVVQAENPGTEPP(A)YK; and IASNFLSAYVVVQAEGGGPDGXLYKV.

Based on the DNA sequences obtained in Examples 8 and 9 below, it is believed that FLKKAKA was an artifact.

Rap GAP DNA sequence may be obtained by methods known in the art, preferably by cDNA cloning of RNA isolated and purified from cellular sources or by genomic cloning. Either cDNA or genomic libraries of clones may be prepared from the DNA fragments generated using techniques known in the art. The preferred procedure is to use oligonucleotide probes to screen cDNA libraries. cDNA libraries can be constructed using techniques known in the art, or can be purchased commercially.

An illustrative procedure for making a cDNA library containing rap GAP sequences may consist of isolating total cytoplasmic RNA from suitable starting material, and further isolating messenger RNA therefrom. The latter can be further fractionated into Poly (A+) messenger RNA, and the messenger RNA can then be reverse transcribed and cloned into a suitable vector to form the cDNA library.

Preferably, the starting material (i.e., tissue, cells) is washed with phosphate buffered saline, and a non-ionic detergent, such as ethylene oxide, polymer type (NP-40) is added in an amount to lyse the cellular, but not nuclear membranes, generally about 0.3%. Nuclei can then be removed by centrifugation at 1,000 x g for 10 minutes. The post-nuclear supernatant is added to an equal volume of TE (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5) saturated phenol/chloroform (1:1) containing 0.5% sodium dodecyl sulfate (SDS) and 10 mM EDTA. The supernatant is re-extracted 4 times and phase separated by centrifugation at 2,000 x g for 120 minutes. The RNA is precipitated by adjusting the samples to 0.25 M NaCl, adding 2 volumes of 100% ethanol and storing at -20°C. The RNA is then pelleted at 5,000 x g for 30 minutes, washed with 70% and 100% ethanol, and dried. This represents the total cytoplasmic RNA. Polyadenylated (Poly A+) messenger RNA (mRNA) can be obtained from the total cytoplasmic RNA by chromatography on oligo (dT) cellulose (J. Aviv et al., 1972, PNAS, 69:1408-1412). The RNA is dissolved in ETS (10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.5) at a concentration of 2 mg/ml. This solution is heated to 65°C for 5 minutes, then quickly chilled to 4°C. After bringing the RNA solution to room temperature, it is adjusted to 0.4 M NaCl and slowly passed through an oligo (dT) cellulose column previously equilibrated with binding buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5). The flow-through is passed over the column twice more, and the column washed with 10 volumes of

binding buffer. Poly (A+) mRNA is eluted with aliquots of ETS, extracted once with TE-saturated phenol chloroform and precipitated by the addition of NaCl to 0.2 M and 2 volumes of 100% ethanol. The RNA is reprecipitated twice, washed once in 70% and then 100% ethanol prior to drying. The poly (A+) mRNA can then be used to construct a cDNA library.

cDNA can be made from the enriched mRNA fraction using oligo (dT) priming of the poly A tails and AMV reverse transcriptase employing the method of H. Okayama et al., 1983, Mol. Cell Biol., 3:280.

Other methods of preparing cDNA libraries are, of course, well known in the art. One, now classical, method uses oligo (dT) primer, reverse transcriptase, tailing of the double stranded cDNA with poly (dG) and annealing into a suitable vector, such as pBR322 or a derivative thereof, which has been cleaved at the desired restriction site and tailed with poly (dC). A detailed description of this alternate method is found, for example, in EP 109,748, published April 13, 1988.

As mentioned above, cDNA libraries are commercially available. A particularly useful library is sold by Clontech Laboratories (Catalog number #L H1008, Palo Alto, CA). It is a lambda gt11 human placenta cDNA library made from total poly (A+) messenger RNA (hereinafter referred to as Clontech cDNA library). Another useful library is the Stratagene human fetal brain cDNA library (hereinafter referred to as "Stratagene cDNA library". It is commercially available from Stratagene Cloning Systems, La Jolla, CA). The Stratagene cDNA library is a λZAP™11 human fetal brain cDNA library made from total poly (A+) messenger RNA.

Other methods can be used to isolate the rap GAP gene. These methods include, but are not limited to, chemically synthesizing the gene sequence itself from a sequence which may, for example, be derived from the amino acid sequence of rap GAP. Alternatively, in vitro translation of selected mRNA followed by functional or immunological assays of the translation products can be used.

The identified and isolated gene can then be inserted into an appropriate cloning vector.

Construction of suitable vectors containing the desired rap GAP coding sequence employs standard ligation and restriction techniques which are well understood in the art. Isolated vectors, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with suitable restriction enzyme(s) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available

restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 μl of buffer solution. In the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about 1-2 hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered form aqueous fractions by precipitation with ethanol followed by chromatography using a Sephadex G-50 spin column. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I, that is, the Klenow fragment, in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 10 mM dNTPs. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of single-stranded portions.

Ligations are performed in 15-30 μl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 μg/ml BSA, 10 mM-50 mM NaCl, and 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C for "sticky end" ligation, or for "blunt end" ligations 1 mM ATP was used, and 0.3-0.6 (Weiss) units T4 ligase. Intermolecular "sticky end" ligations are usually performed at 33-100 μg/ml total DNA concentration. In blunt end ligations, the total DNA concentration of the ends is about 1 μM.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP per μg of vector at 60°C for about 1 hour. Nucleic acid fragments are recovered by extracting the preparation with phenol/chloroform, followed by ethanol precipitation. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

In the constructions set forth below, correct ligations are confirmed by first transforming the appropriate E. coli strain with the ligation mixture. Successful transformants are selected by resistance to ampicillin, tetracycline or other antibiotics,

or using other markers depending on the mode of plasmid construction, as is understood in the art. Miniprep DNA can be prepared from the transformants by the method of D. Ish-Howowicz et al., 1981, Nucleic Acids Res., 9:2989, and analyzed by restriction and/or sequenced by the dideoxy method of F. Sanger et al., 1977, PNAS (USA), 74:5463 as further described by Messing et al., 1981, Nucleic Acids Res., 9:309, or by the method of Maxam et al., 1980, Methods in Enzymology, 65:499.

A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as λ derivatives, pGEM vector, or plasmids such as PBR322 or pUC plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc. Due to the inherent degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the present invention for the cloning for rap GAP.

Such alteration of the rap GAP nucleotide sequence include deletion, additions, or substitution of the different nucleotide residues resulting in a sequence substantially similar to the identified sequence that encodes the same or a functionally equivalent gene product or amino acid sequence. The gene product may contain deletion, additions, or substitution of amino acid residues within the sequence, which result in a silent change, thus producing a bioactive product. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acids and glutamic acids; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, phenylalanine, and tyrosine.

In order to express a biologically active, mature form of rap GAP, an expression vector/host system should be chosen which provides not only for high levels of transcription and translation but for the correct processing of the gene product.

Various animal/host expression vector systems (i.e., vectors which contain the necessary elements for directing the replication, transcription and translation of the rap GAP coding sequence in an appropriate host cell) may be utilized by those skilled in the art. These include, but are not limited to, virus expression vector/mammalian host cell systems (e.g., cytomegalovirus, vaccinia virus, adenovirus, and the like); insect virus

expression vector/insect cell systems (e.g., baculovirus); or nonviral promoter expression systems derived from the genomes of mammalian cells (e.g., the mouse metallothionine promoter).

The expression elements of these vectors vary in their strength and specificities. Depending on the host/vector system utilized, any one of a number of suitable transcription and translation elements may be used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, (e.g., mouse metallothionien promoter) or from viruses that grow in these cells, (e.g., vaccinia virus 5.7 K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Specific initiation signals are also required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire rap GAP gene includes its own initiation codon and adjacent sequences are inserted into the appropriate expression vectors, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon can be provided. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, etc.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing the rap GAP gene and appropriate transcriptional/translational control signals. These methods may include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombinations (genetic recombination).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers, (e.g., zinc and cadmium ions for metallothionein promoters). Therefore, expression of the genetically engineered rap GAP may be controlled. Appropriate cell lines or host systems can also be chosen to ensure the correct modification and processing of the foreign protein expressed.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing chloride, as described by S. N. Cohen, 1972, PNAS (USA), 69:2110, or the $RbCl_2$ method described in Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold

Spring Harbor Press, p. 254 was used for procaryotes. Transfection of Sf9 cells was achieved using a modification of the calcium phosphate precipitation technique (Graham, F.L. et al., 1973, Virology 52:456) as adapted for insect cells (J. P. Burand et al., 1980, Virology 101; E. B. Casstens et al., 1980, Virology 101:311). Additional details regarding transfection of Sf9 cells are described by Summers and Smith in "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", Texas A & M Press: 1986. The baculovirus transfer vectors employed herein are derived from transfer vectors which have been described by G. E. Smith et al., 1983, above. These vectors were originally constructed by cloning the Autographa Californica nuclear polyhedrosis virus (AcNPV) EcoRI-1 fragment containing the polyhedrin gene into the EcoRI site of E. coli plasmid pUC8 as described by Vieira et al., 1982, Gene 19:259-268. A family of plasmids having single BamHI cloning sites at various locations in the polyhedrin gene were created as described by Smith et al., 1983, above. The most used of these, pAc373, has a unique BamHI site 50 base pairs downstream from the polyhedrin cap site, that is to say, 8 base pairs before the polyhedrin ATG translation initiation codon (Luckow and Summers, 1988, Biotechnology, 6:47).

The host cells which contain the recombinant rap GAP coding sequence and which express the biologically active, mature product may be identified by at least four general approaches:

(a) DNA-DNA hybridization;

(b) the presence or absence of "marker" gene functions;

(c) assessing the level of transcription as measured by the expression of receptor mRNA transcripts in the host cell; and

(d) detection of the mature gene product as measured by ligand binding ability, immunoassay and, ultimately, by its biological activity.

In the first approach, the presence of the rap GAP coding sequence inserted in the expression vector can be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are homologous to the rap GAP coding sequence.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the rap GAP coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the receptor coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the receptor sequence under the control of the same or

different promoter used to control the expression of the rap GAP sequence. Expression of the marker in response to induction or selection indicates expression of the rap GAP coding sequence.

In the third approach, transcriptional activity for the rap GAP coding region can be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by Northern blot using a probe homologous to the rap GAP coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the mature protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmunoprecipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active rap GAP gene product. Where the host cell secretes the gene product the cell free media obtained from the cultured transfectant host cell may be assayed for rap GAP activity. Where the gene product is not secreted, cell lysates may be assayed for such activity. Assays such as the assays described herein or the like may be used.

Once a clone that produces high levels of biologically active, mature rap GAP receptor is identified, the clone may be expanded and the rap GAP may be purified as described herein or by using techniques well known in the art. Such methods include affinity purification, chromatographic methods including high performance liquid chromatography, and the like.

Since polymerase chain reaction (PCR) was used in the Examples below for the synthesis/amplification of DNA sequences, the following presents a general description of the PCR technique. PCR is described in U.S. Patent Nos. 4,683,202 and 4,683,195, both of which are hereby incorporated in their entirety. In general, the synthesis/amplification of DNA sequences by PCR involves an enzymatic chain reaction that produces, in exponential quantities, a specific DNA sequence, provided that the termini of the sequence are known in sufficient detail so that oligonucleotide primers can be synthesized which will hybridize to them, and that a portion of the sequence is available to initiate the chain reaction. One primer is complementary to the negative strand, and the other is complementary to the positive strand. As applied to the instant invention, the primers employed are complementary to the 5' end of HP3-12.

The primers are annealed to denatured DNA acid, followed by extension with a suitable DNA polymerase enzyme, such as the large fragment of DNA polymerase I (Klenow), or preferably a DNA polymerase that is stable in the presence of detergents

and nucleotides, which results in newly synthesized plus and minus strands containing the target sequence. Alternatively, a thermostable enzyme may be used which is present in thermostable bacteria. The enzyme may be produced using DNA recombinant techniques as described in EP 258,017, published March 2, 1988. Because the newly synthesized sequences are also templates for the primers, repeated cycles of denaturing, primer annealing and extension results in exponential accumulation of the region defined by the primer. PCR thus produces discrete nucleic acid duplexes of cDNA inserts having termini corresponding to the ends of the specific primers employed.

Although PCR can be performed using a variety of reaction conditions, as described in the references presented above, the preferred reaction conditions are described below in Example 8.

Having described what the applicants believe their invention to be, the following examples are presented to illustrate the invention, and are not to be construed as limiting the scope of the invention. For example, rap GAP obtained from other sources using the purification methods disclosed herein is within the scope of the invention.

Example 1

Purification of Rap GAPm and Indication that Rap GAPm is Different From Cytosolic Rap GAP

The following experiment shows the isolation and purification of rap GAPm from the plasma membrane of differentiated HL60 cells. The purification yielded approximately 3 μg of rap GAPm from 3 x 10^{10} differentiated HL60 cells with about 80% homogeneity, and a molecular weight of about 88,000 as determined by SDS-PAGE analysis after the final purification step of SCE 250 size exclusion HPLC column.

The "Material and Procedure" section presents the preferred method for isolating and purifying rap GAPm. As discussed in the "Results" section, the preferred method was arrived at by first using different buffers to determine how tightly rap GAPm is membrane associated. Second, various detergents were tested to determine the detergent which provides the highest yields of rap GAP activity extracted from the plasma membrane. Finally, the results of the successive steps of the preferred purification steps are discussed.

HL60 cells were grown and differentiated as described in Polakis, P., et al., 1988, J. Biol. Chem., 263:4969-4976. The cells were induced to differentiate by adding dexamethasone and dimethylsulfoxide at 10 mM and 1.2%, respectively,

followed by a 5 day incubation at 37°C. Differentiation was monitored by measuring the number of formylpeptide chemo attractant receptors per cell. Typically, 2-5 x 10^4 receptors per cell were present when the cells were harvested. Cells were subjected to nitrogen cavitation as described in Borregard, N., et al., 1983, J. Cell Biol., 97:52-61, and the lysates were layered over 43% sucrose solution and centrifuged at 25,000 rpm for 1 hour in a swinging bucket SW28 rotor. Plasma membranes were collected from the sucrose interface, washed twice with ice cold 20 mM Tris, pH 8, containing 0.1 M NaCl and 1 mM dithiothreitol and finally resuspended in the same buffer at a protein concentration of approximately 5 mg/ml. Membranes were stored at -80°C until use.

Purification of rap GAP was carried out using plasma membranes isolated from approximately 3 x 10^{10} differentiated HL60 cells. Membranes were suspended at a protein concentration of 5 mg/ml in 20 mM Tris, pH 8.0, containing 0.5% NP-40, 0.5% deoxycholate (Sigma Chemical Co., St. Louis, MO), 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmehtylsulphonyl fluoride (PMSF), and 1 µg/ml each of pepstatin and leupeptin. Following a 30 minute incubation at 4°C the suspension was ultracentrifuged at 2 x 10^5 x g for 60 minutes and the resulting supernatant was adjusted to pH 6.5 with NaH_3PO_4. The extract was applied to 10 ml column of S-Sepharose equilibrated in S-Sepharose buffer (50 mM sodium phosphate, pH 6.5, containing 0.1 % NP-40, 1 mM dithiothreitol, 1 mM EDTA and 1 µg/ml each of leupeptin and pepstatin). The column was washed and eluted with a 150 ml gradient of 0-0.3 M NaCl in the same buffer at a flow rate of 25 ml/hr and fractions of 2.8 ml each were collected. The indicated fractions containing rap GAP activity were pooled, concentrated to 3 ml and dialyzed against Mono Q buffer (20 mM Tris, pH 8.5, 0.1% NP-40, 1 mM EDTA, 0.5 mM dithiothreitol, and 1 µg/ml each of pepstatin and leupeptin). The entire sample was then injected onto a Mono Q HR5/5 FPLC column (5 x 50 mm) (Pharmacia/LKB, Upsula, Sweden) equilibrated in Mono Q buffer. The column was washed with 12 ml of buffer and then eluted with a 40 ml gradient of 0-0.3 M NaCl in the same buffer and fractions of 1 ml each were collected at a flow rate of 1 ml/min. The peak of rap GAP activity was collected, dialyzed against HAP buffer (20 mM Tris pH 8.0, 0.1 M NaCl, 0.1% NP-40, 0.5 mM dithiothreitol, and 1 µg/ml each of leupeptin and pepstatin) and then injected onto a hydroxylapatite HPLC column (Bio-Rad Laboratories, Richmond CA) equilibrated in the same buffer. The column was developed at a flow rate of 1 ml/min with a 40 ml linear gradient of 0-0.2 M potassium phosphate in HAP buffer followed by an isocratic elution with 10 ml of 0.3 M potassium phosphate. Fractions of 1 ml each were collected. The peak fractions from the hydroxylapatite chromatography were combined and concentrated to 0.3 ml and

then injected directly onto an SEC-250 (300 x 7.5 mm) size exclusion HPLC column (Bio-Rad Laboratories, Richmond, CA) equilibrated in 20 mM Tris pH 8.0, 0.2 M NaCl, 0.1% NP-40, 0.5 mM dithiothreitol, 1 mM EDTA and 1 g/ml each of pepstatin and leupetin. Elution was carried out in this same buffer at a flow rate of 1 ml/min and 0.5 ml fractions were collected.

It was estimated that the membrane associated rap GAP activity is approximately 13% of that found in the cytosol from the same cell preparation. The membranes were washed with various buffered solutions to determine how tightly the rap GAP activity was associated with them. The reference was F-Met-Leu-Phe (F-MLP) receptor, which is associated with the plasma membrane of differentiated HL60 cells. Niedel, J.E., et al., 1980, PNAS (USA), 77:1000-10004. ([3H] F-MLP was purchased from DuPont NEN, Boston, MA.) Plasma membranes (100 g total protein) from differentiated HL60 cells were suspended in 20 mM Tris, pH 8.0, or buffer containing: 1 M NaCl; 1 M LiCl + 20 mM EDTA; 3 M urea; or 1% NP-40 and incubated at 4 C for 30 minutes. Samples were centrifuged and the pellets were resuspended in 25 1 Tris buffer and assayed for rap GAP activity and formylpeptide binding as described in "Materials and Procedures". The results are expressed as a percentage of the amount of association of rap GAP with the plasma membrane determined for membranes incubated in Tris buffer only. The results showed that the rap GAP activity was quantitatively retained in the membrane following washing with buffered 1 M NaCl or 1 M LiCl containing 20 mM EDTA, but was lost when membranes were treated with 1% NP-40 or 3 M urea (Figure 2). A similar pattern was observed for the binding of radiolabelled F-MLP to these same membrane preparations (Figure 2).

Next, various buffered detergent solutions was tested and it was found that a combination of 0.5% NP-40 and 0.5% deoxycholate produced the highest yields of rap GAP activity extracted from the plasma membranes. The extract markedly stimulated the GTPase activity of rap (Figure 3A). However, the degree of stimulation was difficult to calculate as rap did not exhibit detectable GTPase activity in the absence of GAP under these conditions (Figure 3A). The rap GAP activity increased linearly with added protein but then deviated from linearity if more than approximately 70% of the GTP bound to rap was hydrolyzed (Figure 3B).

The above experiment showed that rap GAPm could be solubilized from the plasma membrane in an active form. Therefore, the preferred purification method uses successive steps of column chromatography in the presence of detergent. Due to the inability of most proteins to adsorb to S-Sepharose cation exchange resin at pH 6.5, a high degree of purification of rap GAP was achieved with this first step. Greater than

95% of the total protein applied to this resin was eluted in the flow through fractions, however, the rap GAP activity was quantitatively adsorbed and a peak over 50% of the activity was recovered upon elution of the column with NaCl (Figure 5).

The fractions containing the highest levels of rap GAP activity, fractions 42-48, were pooled and chromatographed on a Mono Q FPLC column from which two peaks of activity were eluted (Figures 6A &6B). The second peak, eluting within the NaCl gradient, was not further purified.

The first peak of activity, fractions 10 to 11 from the Mono Q FPLC chromatography eluted during the isocratic wash were pooled for further purification. These fractions were applied to an hydroxylapatite HPLC column. The column was developed with a gradient of potassium phosphate and a single peak of rap GAP activity was eluted (Figure 7A). SDS-PAGE run under reducing conditions revealed the presence of two major polypeptides with molecular weights of approximately 88,000 and 60,000 in the fractions containing the rap GAP activity (Figure 7B).

The rap GAP eluted from the hydroxylapatite column, from fractions 17-19, was combined and applied to an SEC-250 size exclusion HPLC column. The rap GAP activity eluted as a single peak which corresponded closely to the elution of the polypeptide of about 88,000 molecular weight (Figure 8A). The commigration of the polypeptide of about 88,000 molecular weight with the rap GAP activity was seen on the hydroxylapatite and SEC-250 column chromatography profiles. The purification of rap GAPm was repeated three times and a polypeptide of about 88,000 molecular weight was identified in the final chromatography steps from all three preparations. The crude, freshly solubilized rap GAPm has a mobility similar to that of the highly purified rap GAPm on size exclusion chromatography (compare Figures 8A and 4A).

A protein of about 60,000 molecular weight contained in the hydroxylapatite pool, eluted in fractions 15 and 16, were outside of the area of the GAP activity peak. A minor polypeptide with molecular weight of in fractions 18 and 19 in SDS-PAGE analysis (Figure 8B). However, the elution pattern of this protein did not correspond closely to the rap GAP activity. Moreover, the protein of about 64,000 molecular weight was not present in the rap GAP fractions eluted from the hydroxylappatite column (Figure 7B) suggesting that it is generated between chromatography steps or during sample manipulation.

A summary of the purification is shown below in Table 1.

Table 1

Purification of rap GAP from HL60 Cell Plasma Membranes

	Total Protein (a)	Total Activity (b)	Specific Activity	Fold Purification	Yield
	mg	units (x 10^{-3})	units (x 10^{-3})/mg		%
Extract	111	25.5	0.23	1	100
S-Sepharose	1.4	8.8	6.3	27.4	34
Mono Q	0.022	2.11	95.9	417	8.2
Hydroxyl-apatite	0.003	1.22	406.7	1768	4.8

(a) Except for the hydroxylapatite pool, the total protein in the extract and pooled chromatography fractions was estimated by staining with Amido Black as described in Schaffner, W., et al., 1973, Anal. Biochem., 56:502-504, the standard was bovine serum albumin. In the case of the hydroxylapatite pool, the total protein was estimated by comparing silver staining intensities to known quantities of standard proteins on SDS-PAGE.

(b) One unit of activity is defined as the hydrolysis of 50% of the [γ-^{32}p]GTP bound to rap relative to buffer control following a 10 minute incubation at 23 C.

Based on the calculations shown in Table 1, it is clear that rap GAP is a relatively minor protein component in the plasma membrane from HL60 cells. Rap GAP is estimated to constitute less than 0.05% of the total membrane protein.

Example 2

Rap GAPm is Different From Cytosolic Rap GAP

Rap GAP protein found in the plasma membrane is different from that found in the cytosol of the cells. This is demonstrated by subjecting both the freshly prepared membrane derived and cytosolic rap GAP to size exclusion chromatography under identical conditions. The cytosolic preparation was equilibrated in the detergent extraction buffer used to solubilize the rap GAPm prior to its injectiononto the column. The two forms of rap GAP migrated with radically different mobilities on the SEC-250 HPLC column (Figure 4A). The stokes radii of the cytosolic and membrane derived rap GAPs were estimated to be 54 and 36 , respectively (Figure 4B).

Additionally, the cytosolic rap GAP was purified under conditions identical to those employed for the rap GAPm. Although the cytosolic rap GAP could not be

purified to homogeneity, its elution profiles on S-Sepharose and Mono-Q chromatography differed from those of rap GAPm.

Example 3

Isolation and Purification of Rap GAPb

The following experiment purified rap GAPb from bovine brain membranes and yielded approximately 40 g of rap GAPb per 8.4 grams of membrane protein used. The purified rap GAPb was about 50% homogeneous and has a molecular weight of about 88,000 as determined by reduced SDS-PAGE analysis after the final purification step PW-5 HPLC column. (Figure 9B). The isolation and purification of rap GAPb was carried out as follows.

Crude bovine brain membranes were prepared essentially as described in Waldo, G.L., et al., 1987, Biochem J., 246:431-439. Membranes (8.4 gram total brain membrane protein) were washed twice with 20 mM Tris, pH 8.0, containing 0.2 M NaCl and finally resuspended using a polytron in 350 ml of 40 mM Tris, pH 8.0, to which was added an equal volume of 1.0 % NP-40, 1.0 % deoxycholate, 0.5 mM dithiothreitol,1 mM EDTA, 0.5 mM phenylmehtylsulfonyl fluoride, and 1 g/ml each of pepstatin and leupeptin. Following a 1 hour incubation with stirring, the insoluble material was removed by ultracentrifugation at $2 \times 10^5 \times g$ for 60 minutes. The supernatant was pH adjusted to 6.5 with NaH_3PO_4 and ultracentrifuged again. The resulting clear supernatant was applied at a flow rate of 30 ml/hr to 100 ml column (2.5 x 20 cm) of S-Sepharose equilibrated in S-Sepharose buffer (as described in Example 1). The column was washed with 100 ml of this buffer and then eluted at a flow rate of 30 ml/hr with a 500 ml gradient 0-0.5 M NaCl in the same buffer and fractions of 7 ml each were collected. The rap GAP activity eluting between 0.17 and 0.25 M NaCl was pooled, concentrated to 15 ml, and then applied to a 500 ml column (2.5 x 100 cm) of Sephacryl S-300 equilibrated in 20 mM Tris, pH 8.0, containing 0.1 % NP-40, 0.1 M NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 1 g/ml each of pepstatin and leupeptin. The column was eluted at a flow rate of 40 ml/hr and fractions of 7 ml each were collected. The rap GAP activity eluted as a single symmetrical peak between 280 and 310 ml of elution buffer. The peak fractions (28 ml) were diluted to 84 ml with Mono Q buffer (as described in Example 1) and then loaded onto a Mono Q HR 5/5 FPLC column equilibrated in Mono Q buffer. The column was eluted at a flow rate of 1 ml/min with a linear gradient of 0-0.5 M NaCl in Mono Q buffer and fractions of 1 ml each were collected. A single peak of activity eluted between 0.3-0.35 M NaCl and was collected and dialyzed against S-Sepharose buffer over night. This material was injected onto a cation exchange SP-5-PW HPLC column (75 x 7.5 mm) (commercially

available from Bio-Rad Laboratories, Richmond, CA) equilibrated in S-Sepharose buffer. The column was washed with 10 ml of buffer and then eluted at a flow rate of 1 ml/min with a 60 ml gradient of 0-0.5 M NaCl in S-Sepharose buffer and fractions of 1 ml each were collected.

Example 4

Isolation and Sequencing of Rap GAPb

Rap GAPb was isolated and sequenced as follows.

Proteins resolved by reduced SDS-PAGE were visualized by briefly staining the gels with Coomassie Blue. The protein bands corresponding to molecular mass of about 88 kD were excised and following a 5 minute equilibration of the gel slice in electroelution buffer (Tris-glycine (20-192 mM), 0.1% SDS (w/v)), the rap GAP was electroeluted using a Bio-Rad model 422 electro-eluter according to the manufacturers' instructions. The eluted rap GAP was precipitated with chloroform and methanol as described in Wessel, P., et al., 1984, Anal. Biochem., 138:141-43. For cyanogen bromide digestions the rap GAP was dissolved in 50 1 of 70% formic acid containing 30 mg/ml cyanogen bromide and incubated in the dark at room temperature for 24 hours. The formic acid was evaporated and the peptides were dissolved in SDS-PAGE sample buffer and then subjected to SDS-PAGE and electroblotting. The procedures for SDS-PAGE and electroblotting were as described previously under "C. Procedure for Purification of Rap GAP," and "II. Rap GAP Assay", respectively. The peptides were detected on the electroblots by brief staining with Coomassie blue, and the excised bands were applied directly to an Applied Biosystems mode 470A amino acid sequencer.

The two peptides derived or generated from rap GAPb have the following amino acid sequences: F(G)VSTKLPFT(W)DXA(Q)QL, and FLKKAKA, respectively, (wherein X denotes an unknown residue and the parenthesis indicate uncertainty in the enclosed residue).

Further experiments were conducted to obtain additional purified rap GAP_b and the following partial sequences: IASNFL(P)AYIVVQAENPGTEPP(A)YK; and IASNFLSAYVVVQAEGGGPDGXLYKV.

Based on the DNA sequences obtained in Examples 8 and 9 below, it is believed that FLKKAKA was an artifact.

Example 5

Characterization of Rap GAPm and b

The purified rap GAPm was tested for its activities towards the GTP bound forms of ras p21, rhoB, G25K, and rac 1. The assays were similar to that of rap assay described previously. It was observed that greater than 95% of the GTP bound to rap was hydrolyzed relative to the buffer control, but no detectable activity was observed with ras p21, rhoB, or G25K. Approximately 13% of the GTP bound to rac-1 was hydrolyzed relative to the buffer control. However, the low level of activity with rac-1 was likely due to the trace amounts of rap protein previously detected in these preparations by immunoblot analysis. Since the rap GAP assay used in this study was based on the quantitation of [γ-^{32}P]GTP remaining bound to rap, an increase in the dissociation of [γ-^{32}P]GTP could be interpreted as an increase in GTP hydrolysis. To address this, the purified rap GAP preparations were also tested using [α-^{32}P]GTP bound to rap. No loss of bound radioactivity was noted in response to rap GAP.

Further, neither rap GAPm nor rap GAPb stimulates the dissociation of [γ-^{32}P]GTP and [γ-^{32}P]GDP from rap. The assays were similar to that of [γ-^{32}P]GTP assay described previously. This indicates that rap GAPm is distinct from the GTPase activating proteins specific for ras p21 and rho B. The activities of rap GAPm and rap GAPb were killed by heat boiling.

Example 6

Production of Antibodies to Rap GAP

Antibodies to rap GAP are produced using standard procedures known in the art. For example, antibodies are produced by injecting a host animal such as rabbit, rat, goat, mouse, etc., with the rap GAP or fragments thereof, alone or conjugated to an appropriate carrier if required to elicit an antibody response. The rap GAP can be excised from the reduced SDS-PAGE, combined with an adjuvant, for example, complete Freund's adjuvant, and used to immunize the host animals. It will be appreciated by those skilled in the art that monoclonal antibodies (MABs) to the above rap GAP are produced by means of the hybridoma technique.

Example 7

Synthesis of Peptide Sequences

The peptides containing the partial sequences of rap GAP may be synthesized by methods well known in the art. The preferred method of peptide synthesis is the

solid-phase method, described in more detail in Merrifield, R.B., 1985, Science, 232:341-347, on a Biosearch 9500 automated peptide machine, cleaved with hydrogen fluoride and purified by preparative HPLC using a Waters Delta Prep 3000 instrument, on a 15-20 μm Vydac CH Prep PAK column. An alternative method is by means of ABI Automatic Synthesis. Thus, peptides of the following sequences F(G)VSTKLPFT(W)DXA(Q)QL; IASNFL(P)AYIVVQAENPGTEPP(A)YK; and IASNFLSAYVVVQAEGGGPDGXLYKV (wherein X denotes unknown residue and parenthesis indicate uncertainty in the enclosed residue), are respectively synthesized. These synthetic peptides can be used to raise the polyclonal and monoclonal antibodies to rap GAP. For example, before injection into the host animals, these peptides may be conjugated with keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA). The conjugation is achieved via the sulfhydryl group in the cysteine residue. The methods for such conjugation and the production of antibodies using the conjugate thus produced are described in U.S. Patent No. 4,762,706, issued August 9, 1988, to McCormick, et al., hereby incorporated by reference. It will be appreciated by those skilled in the art that monoclonal antibodies (MABs) to the above rap GAP are produced by means of the hybridoma technique. These monoclonal antibodies are useful for purifying rap GAP as in Example 10.

Example 8

Cloning of Rap GAP

A full length cDNA sequence that encodes rap GAP was obtained as follows:

First, partial cDNA sequences were identified in a cDNA library using a synthesized oligonucleotide probe, designated BR67, based on the partial rap GAP amino acid sequence, AYIVVQAENPGTEPP, described in Example 4 and codon redundancies thereto.

The DNA sequence for BR67 is as follows:

5'-GGGGGCTCTGTGCCAGGGTTCTCAGCCTGCACCACAATGTAGGC-3'

The cDNA library used was Clonetech cDNA library described supra under "IV. Identification and Isolation of rap GAP Sequences." One such partial cDNA sequence, referred to as HP3-12 (the DNA sequence of HP3-12 is indicated in Figure 10) was subcloned and sequenced. Knowledge of its DNA sequence led, in turn, to additional probes that were used to screen the Stratagene cDNA library (described supra under "IV. Identification and Isolation of Rap GAP Sequences") for longer cDNA inserts, eventually yielding the full length clone, clone HUB10A (shown in Figure 10). The procedures for obtaining the longer clones are as follows:

Synthetic oligonucleotides were prepared by the triester method of Matteucci et al., 1981, J. Am Chem. Soc., 103:3185 or using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 0.1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles γ^{32}P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

As described above, the oligonucleotide probe designated BR67 was used to screen the Clontech cDNA library. While various procedures are known, a description of the preferred procedure follows. The library was plated at about 50,000 plaques perplate using 5 plates. Thus, about 250,000 plaques were initially screened using the plaque hybridization procedure. Each 150 mM plate was replicated onto duplicate nitrocellulose filter papers (S & S type BA-85). DNA was fixed to the filter by sequential treatment for 5 minutes with 0.5 N NaOH plus 1.0 M NaCl; 1.5 M NaCl plus 0.5 M Tris-HCl pH 8; and 20 mM Tris plus 2 mM EDTA pH 8. Filters were air dried and baked at 80°C for 2 hours.

Duplicate nitrocellulose filters were prehybridized at 42°C for 2-4 hours with 10 mls per filter of DNA hybridization buffer (5 x SSC, 5 x Denhardt's solution, 50 µg/ml salmon sperm DNA, 50 mM sodium Phosphate, 0.1% SDS) with 30% formamide. (5 x Denhardt's solution contains polyvinylpyrrolidone, and 1 x 0.02% each of Ficoll and bovine serum albumin). The buffer was removed and the samples were hybridized with the kinased probe under conditions which depend on the stringency desired. About 1 to 2 x 10^6 cpm/ml total was used. Typical moderately stringent conditions employ a temperature of 42°C plus 50% formamide for 24-36 hours with 1-5 ml/filter of DNA hybridization buffer containing probe. For higher stringencies high temperatures and shorter times were employed. The preferred hybridization conditions consisted of a first lift under high stringency conditions (16 hours at 42°C with 50% formamide in hybridization buffer) and a second lift under low stringency condition (16 hours at 42°C with 30% formamide in hybridization buffer) overnight. About 1 x 10^6 cpm/ml was used. The filters were washed twice, 30 minutes each wash, with 2 x SSC/0.1% SDS and 0.2 x SSC/0.1% SDS at 65°C and then air dried. Finally, the filters were autoradiographed at -70°C for 72 hours with intensifying screens.

The autoradiographic results revealed 106 positives of which 27 were pursued for further characterization. The positive recombinant bacteriophages were purified, one of which was termed HP3-12. The phage DNA was isolated as follows. HP3-12 was plated at high density on a lawn of E. coli strain Y 1090 (r-) (commerically

available from Clontech). Following lysis of the E. coli, phage particles were eluted into S M buffer (0.1 M NaCl 8.1 mM $MgSO_4$ 50 mM Tris - HCl pH 7.5 0.01% Gelatin) by covering the E. coli with buffer and incubating the plate in the cold for 6 or more hours. The lysate containing phage particles was centrifuged at 11,500 x g for 20 minutes to remove cellular debris, and the resulting supernatant titered using standard techniques. Finally, phage DNA was isolated by the procedure of Maniatis et al., supra.

HP3-12 was subcloned into a suitable vector in order to obtain DNA sequence. Although DNA can be cloned into a variety of vectors, in the instant invention it was subcloned into the pGEM vector (Promega, Madison, WI).

The cDNA insert for HP3-12 was subcloned into pGEM as follows: HP3-12 λ DNA was digested with EcoR1 which produced two fragments, the cut λ DNA and an insert of about 3.0 kb. The insert was isolated using low melt agarose (SeaPlaque®, FMC Corp., Rockland, ME) and ligated into pGEM using techniques described in Struhl K., 1985, BioTechniques, 3(6):453. The pGEM vector is designed such that when it was transformed into an E. coli lacking a portion of the lac region (i.e. DH5α) and plated onto an indicator plate (i.e. MacConkey's Agar, commerically available from Difco Co., Detroit, MI, Cat. # D-781138). The E. coli colonies containing the vectors with inserts appear white; whereas those without the inserts appear red.

The ligated vector was transformed into competent E. coli DH5α and plated onto MacConkey's Agar with 50 μg/ml Ampicillin. White recombinnants were picked into liquid media containing L-broth and 50 μg/ml of ampicillin, and the plasmid DNA was isolated by Qiagen preparation (Qiagen, Studio City, CA. The isolation method is disclosed in the instruction manual accompanying the Qiagen preparation). One subclone of HP3-12, designated 121, was found to contain the 3.0 kb fragment.

Nucleotide sequence of HP3-12 (Figure 10 shows the sequence of HP3-12) was determined by primed DNA synthesis in the presence of dideoxynucleotide triphosphates.

HP3-12 was sequenced and several oligonucleotides based on the sequence were synthesized. The sequences of these oligonucleotides are shown below:

BR116: 5'-GGTTGCACTCGAGCTTCACCTTGGT-3'

BR119: 5'-AAGCACTTTCTCGGCAAGGA-3'

BR120: 5'-GAACCGATCCACATTGACGT-3'

BR149: 5'-AACTTTGCCATCTGGACAACATTAGGGAACTCGGTGAGGCAGGAGATGG-3'

A radioactively labelled 233-bp PCR product was generated as follows: 1-10 ng HP3-12 cDNA EcoR1 fragment was used as template along with 2 μM of dATP and of dTTP, respectively; 1 μM of dCTP and dGTP, respectively; 1 μM (100 μCi) of [α-32P]dCTP and [α-32P]dGTP, respectively; 10 pmoles of upstream (i.e. BR119) and downstream (i.e. BR120) primers, respectively; 1 x PCR buffer (50 mM KCl, 20 mM Tris-HCl 8.0, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA) and 1 unit Taq polymerase (Perkins-Elmer - Cetus Corp., Emeryville, CA). Reaction cycle was 95°C-30s, 60°C-30s, 72°C-30s; 20-25 cycles (using Thermal Cycler, Perkins-Elmer - Cetus Corp.). The resulting product was purified through a spin column.

Either an oligonucleotide probe or the labeled PCR product was used to rescreen the Clontech cDNA library or to screen the Stratagene cDNA library. Filters were hybridized as follows: those probed with BR116 were hybridized at 42°C in hybridization buffer with 35% formamide, those probed with the PCR product described above or the oligonucleotide BR149 were hybridized at 42°C in hybridization buffer with 50% formamide. All filters were washed twice, 30 minutes each wash, with 2 x SSC/0.1% SDS and 0.2 x SSC/0.1% SDS at 65°C and then air dried. Finally, the filters were autoradiographed at -70°C for 72 hours with intensifying screens.

Autoradiography of the filters revealed that of the ten positives from the Clontech cDNA library probed exclusively with BR116, eight were determined to be the same as HP3-12. Of the 5 positives obtained from the Stratagene cDNA library, 4 were determined to be the same size and contained 400 bp more sequence than HP3-12 (as determined by PCR analysis). The plasmid containing the cDNA insert was excised according to the method described in the Stratagene user manual. One of the clones was designated HuB10A and was 3.4 kb in length. (Figure 10 shows the sequence of HuB10A).

Example 9

Expression of Rap-GAP

The 3.4 kb HuB10A EcoR1 cDNA insert was subcloned into the Baculovirus transfer vector, pAcC13 to generate pAcRG9. The EcoR1 fragment from HP3-12 was cloned into the baculovirus transfer vector pAcC13, and was designated pAcRG1. The pAcC13 vector was derived from pAcC12 vector which in turn was derived from the pVL941 vector. The construction of pVL941 was disclosed in Luckow, V. A. et al., 1989, Virology, 170:31. The construction of pAcC12, a polylinked derivative of pVL941, was disclosed in Quilliam, L.A., et al., 1990, Mol. Cell. Biol., 10(6):2901.

pAcC12 was derived from pVL941 by deleting the unique EcoRI site at 7.15 kilobases and introducing a polylinker sequence (GGTACCCGGGCTGCAGAATTCTAGATCTCGAGCTCCATGGTGGATCC) at the unique BamHI insertion site. Id.

pAcC13 was derived from pAcC12 by replacing the KpnI fragment between 4 and 4.5 map units with a synthetic oligonucleotide containing the polylinker sequence 5'- GTACCAGATCTGCAGAATTCTAGAGGATCCTGATCAGCTAGAGAGCTC GCGGCCGCCCGGGCCGTAC-3'. The construction of pAcC13 is shown by Munemitsu, S., et al., 1990, Mol. Cell. Bio., 10(11):5977.

About 2 μg of either plasmid was transfected into 2 x 10^5 Sf9 cells, the cells grown for 4 days, isolated by centrifugation, and cell extracts made by solubilizing the cell pellet. The preferred solubilization solution consists of 20 mM Tris, pH 8.0, containing 0.5% NP-40, 0.5% deoxycholate (Sigma Chemical Co., St. Louis, MO), 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmehtylsulphonyl fluoride (PMSF), and 1 μg/ml each of pepstatin and leupeptin. The extract was centrifuged for 15 minutes at 15,000 x g and aliquots diluted into GAP assay buffer, and assayed for GAP activity as described above.

Methods for growing Sf9 cells are well known in the art, and detailed procedures for their cultivation can be found in M. Summers and G. Smith in "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", Texas Agricultural Experiment Station, Bulletin No. 1555 (May, 1987) or in EPO 127,839 to G.E. Smith and M. D. Summers.

It is important to note that baculovirus can be recovered from Sf9 cells transfected with the above described transfer vectors using the techniques described by Summers and Smith, above. Such virus can be employed to transform cells directly with the appropriate rap GAP clone.

100 μl of Sf9 cell suspension was pelleted and resuspended in 5 ml 20 mM Tris, pH8, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF. The mixture was then ultracentrifuged at 100,000 x g for 60 minutes. The supernatant was adjusted to pH 6.5 using sodium phosphate. The resulting mixture was then loaded onto 10 ml S-Sepharose, pH 6.5, and eluted with 100 ml 0-0.5 M NaCl, and 2 ml fractions were collected. The S-Sepharose chromatography was conducted as described in the purification of rap GAP from bovine brain (i.e. Example 1).

Expression levels from the pAcRG1 construct was low and therefore another construct was engineered to introduce an initiating methionine and the epitope for the Glu-Glu antibody [EEEEYMPME], subsequent to the EcoR1 site. The resulting 5'

amino acid sequence would read MEEEEYMPMEGIRA [PTTK . . .]. This construct was designated pAcRG4 and was used for subsequent purifications.

The EcoR1 fragment from HuB10A was cloned into pAcC13 as described above and designated pAcRG9. Expression of rap-GAP from both these constructs (i.e. pAcRG4 and pAcRG9) were very high in that the rap GAP protein represented more than 50% of the total protein recovered from the cell lysate. The rap GAP activity, as assayed using the preferred method described above in section II, subpart C, "Procedure for Rap GAP Assay", was over 10,000-fold higher in lysates prepared from AcRG4 infected cells compared to lysates from Sf9 cells infected with virus containing deleted polyhedrin promoter only. AcRG9 infected cells produced similar result. SDS-PAGE gel analysis of transformed cell lysates revealed a broad band of 80-110 kD (Figure 11, lane L). This diffuse staining pattern appears to be composed of several bands that were partially resolved and comigrated with the rap GAP activity when chromatographed on an S-Sepharose column (Figure 11)

The exceptionally high yield of rap GAP protein from the transformed Sf9 cells resulted in the purification (according to the method disclosed in Example 1 above) to near homogeneity of approximately 7 mg of rap GAP from 100 ml of Sf9 cell suspension.

Figure 12 shows that antibodies, raised against a synthetic peptide based on a partial sequence of rap GAP: GQTSEEELFSTNEES (designated S in Figure 10), recognized and bound to rap GAP protein. The preparation of the synthetic peptide, rabbit antibodies, and the Western blot was as described in "II. Rap GAP Assay", above.

The methods for isolating cytosolic and membrane recombinant rap GAP are as follows. The pellet was solubilized in 20 mM Tris, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, pepstatin and leupeptin. The resulting mixture was ultracentrifuged at 100,000 x g for 60 minutes. The resulting supernatant was the cytosolic fraction. To isolate the membrane fraction, the resulting pellet was washed once with the above buffer and recentrifuged. The pellet was then solubilized in the above buffer containing 0.5% NP40 and 0.5% deoxycholate and recentrifuged. The resulting supernatant was the membrane fraction.

Example 10

Methods of Purifying Rap GAP Using Monoclonal Antibodies to Rap GAP

The above MABs to rap GAP can be used to isolate and purify rap GAP. For example, an efficient immunoaffinity method for isolating membrane associated rap GAP involves the use of biotinylated MABs (b-MABs) and streptavidin-agarose. The general method is outlined in Updyke, T.V., et al., 1986, Methods in Enzymology, 121:717-725, entitled "Immunoaffinity Isolation of Membrane Antigens with Biotinylated Monoclonal Antibodies and Streptavidin-Agarose" (hereinafter referred to as Updyke).

When covalently linked to agarose, streptavidin has high binding capacity for b-MABs and low nonspecific binding characteristics. Streptavidin-agarose can therefore be used to efficiently bind immune complexes found between b-MABs and detergent-solubilized membrane rap GAP. Id.

Updyke presents methods for preparing the streptavidin-agarose matrix and determining the amount of streptavidin linked to the agarose matrix using [^{14}C] biotin binding assay. The methods for purifying and biotinylating the MABs to rap GAP are essentially as presented in Updyke. The preferred MABs are those produced by hybridomas, described in the previous section, grown in serum free medium. Membrane detergent extracts are incubated with the biotinylated MABs and the mixture is then passed over streptavidin-agarose. The nonadsorbed proteins are washed off the resin and adsorbed proteins are eluted by denaturation. The eluted proteins are analyzed by SDS-PAGE and staining with Coo Blue or silver.

Example 11

Methods for Identifying Anti-Cancer Therapeutics

The rap GAP described herein can be used to identify anti-cancer therapeutics, particularly those that are effective against ras related tumors. It is known that rap reverts ras transformed cells. Kitayama, H., et al., 1989, Cell, supra. It has been shown that rap GAP does not stimulate ras p21 GTPase activity, nor does ras p21 GAP stimulate the GTPase activity of rap. Kikuchi, A., et al., 1989, J. Biol. Chem., 264:9133-9136. Significantly, it has also been shown that rap·GTP binds ras GAP; but rap·GDP does not bind ras GAP (unpublished manuscript, Frech, M. & Wittinghofer, A., et al.). Mutant rap Val 12 contains an amino acid sequence corresponding to mutant ras Val 12 which is insensitive to ras GAP, i.e., the mutant ras Val 12 is not stimulated by ras GAP. Ras Val 12 is a mutant of wild-type ras Gly 12. Rap Val 12 has been transfected into the human tumor cell line HT 1080 which contained activated oncogenic ras gene. It was then shown that rap Val 12 was about

ten times better than the wild-type rap Gly 12 at reverting the ras oncogenic phenotype. (Speech by M. Noda, at U.S. Japan Study Group, Hawaii, January 23, 1990). Without wishing to be bound by any theory or applicants' proposed models, applicants propose Models A and B regarding the role rap GAP plays in the reversion of ras transformed cells.

Model A proposes that it is the rap·GTP, when bound to ras GAP, which causes the reversion of the ras transformed cells. In this model, the rap GAP serves only to stimulate the hydrolysis of GTP, i.e. it serves only to stimulate the conversion of rap·GTP to rap·GDP. In order to revert a ras-transformed cell, the rap must be maintained in the rap·GTP form, this can be achieved by preventing rap GAP from binding to rap·GTP.

Thus, a chemical which prevents rap GAP from binding to rap·GTP can serve as an anti-cancer therapeutic. For example, this chemical can be identified in the following assay. In this assay, "X" denotes the chemical to be screened. "X" is added to rap GAP under experimental conditions essentially similar to the rap GAP assays described previously, in which the production of rap·GDP is assayed. After an appropriate incubation period, rap·GTP is added to the mixture. If "X" prevents or inhibits the production of rap·GDP, then X can serve as an anti-cancer therapeutic.

Model B, unlike Model A, proposes that it is the rap GAP in the rap GAP-rap·GTP complex which is responsible for the reversion of ras-transformed cells. That is, rap·GTP reverts the transformed phenotype by interacting with rap GAP as an effector target. In this model, an anti-cancer therapeutic chemical is one which fulfil the requirement of rap·GTP in the rap GAP-rap·GTP interaction. It is proposed that the downstream product(s) of rap GAP-rap·GTP interaction serves to regulate and revert ras oncogenic behavior.

A successful anti-cancer therapeutic would be one which can substitute for rap·GTP in interacting with rap GAP to revert ras-transformed cells. The assay will involve introducing the potential anti-cancer therapeutic "X" into ras transformed cells, and assaying for increased production of the downstream product or decrease in the upstream target (precursor of the downstream product). Alternatively, the following assay may be used if the downstream substrate of rap GAP-rap·GTP interaction is known. The in vitro assay would involved first labelling the substrate using methods known in the art, e.g., radiolabelling. The labelled substrate is then mixed with rap GAP, and chemical "X" that is to be screened. The production of labelled product is then assayed. "X" can serve as an anti-cancer therapeutic if labelled products are obtained.

Deposit of Biological Materials: The following plasmid has been deposited with the American Type Culture Collection (ATCC), 12001 Parklawn Drive, Rockville, Maryland 20852 (USA) pursuant to the provisions of the Budapest Treaty.

Designation	Deposit Date	ATCC No.	CMCC No.
pAcRG9			3956

Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Also, the present invention is not to be considered limited in scope by the deposited recombinant transfer vector or plasmid, since the deposited vector or plasmid is intended only to be illustrative of particular aspects of the invention.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

WE CLAIM:

1. A substantially pure rap GAP.

2. A substantially pure protein comprising an apparent molecular weight of about 80,000 - 90,000 as assessed by SDS-PAGE under reducing conditions, or fragments derived therefrom, that stimulates GTPase activity of rap.

3. The molecule of claim 2, wherein the molecule has a homogeneity of about 50% or above, as assessed by SDS-PAGE under reducing conditions.

4. The molecule of claim 3, wherein the molecule is purified from the plasma membrane of HL60 cells.

5. The molecule of claim 3, wherein the molecule is purified from bovine brain membrane.

6. A method of purifying a molecule that stimulates GTPase activity of rap, from a solution containing the same, comprising the steps of:

a) contacting the solution with cation exchange chromatographic material for a time sufficient for the molecule to bind to the material;

b) forming a first eluate containing the molecule by eluting the molecule from the cation exchange chromatographic material by contacting the chromatographic material with an effective aqueous salt solution;

c) identifying fractions in the first eluate having the molecule;

d) forming a second eluate by passing the fractions of the first eluate containing the molecule through a size exclusion column;

e) identifying fractions of the second eluate having the molecule, and reducing the salt concentration present in the fractions to be compatible with anion exchange chromatography;

f) forming a third eluate by contacting the fractions of the second eluate containing the molecule with an anion exchange chromatography material for a time sufficient for the molecule to bind to the material, and eluting the molecule from the material with an effective aqueous salt solution;

g) identifying fractions of the third eluate having the molecule, and reducing the salt concentration present in the fractions to be compatible with cation exchange chromatography;

h) forming a fourth eluate by contacting the fractions of the third eluate containing the molecule with a second cation exchange chromatography material for a time sufficient for the molecule to bind to the material, and eluting the molecule from the material with an effective aqueous salt solution; and

i) identifying fractions of the fourth eluate having the molecule.

7. The method of claim 6, wherein the molecule is a membrane bound molecule, and the steps further comprise an initial step of isolating the molecule from the membrane.

8. The method of claim 6, wherein the purification is conducted in solutions containing one or more protease inhibitors at concentrations that effectively inhibit proteolysis of said molecule.

9. The method of claim 8, wherein the molecule is present in a solution comprising a reducing agent in an amount effective to preserve the activity of said molecule.

10. The method of claim 9, wherein the purification is conducted in solutions containing one or more metal ion chelators at concentrations that prevent substantial loss of activity of said molecule.

11. The method of claim 6, wherein the solution comprises about 0.5% NP-40, 0.5% deoxycholate, 0.5 mM DTT, 1 mM EDTA, 0.5 mM PMSF, 1 μg/ml pepstatin and 1 μg/ml of leupeptin.

12. The method of claim 6, wherein the first cation exchange chromatographic material is S-Sepharose.

13. The method of claim 6, wherein the anion exchange chromatographic material is Mono Q HR 5/5 FPLC.

14. The method of claim 6, wherein the second cation chromatographic material is PW-5 HPLC.

15. The method of claim 6, wherein the size exclusion column is Sephacryl S-300.

16. The method of claim 6, wherein the first cation, anion, and second cation exchange chromatographic materials and size exclusion column, are S-Sepharose, Mono Q HR 5/5 FPLC, PW-5 HPLC, and Sephacryl S-300, respectively.

17. A method of purifying a molecule that stimulates GTPase activity of rap, from a solution containing the same, comprising the steps of:

a) contacting the solution with cation exchange chromatographic material for a time sufficient for the molecule to bind to the material;

b) forming a first eluate containing the molecule by eluting the molecule from the cation chromatographic material by contacting the chromatographic material with an effective aqueous salt solution;

c) identifying fractions in the first eluate having the molecule, and reducing the salt concentration present in the fractions to be compatible with anion exchange chromatography;

d) forming a second eluate by contacting the fractions of the first eluate containing the molecule with anion exchange chromatographic material for a time sufficient for the molecule to bind to the material, and eluting the molecule from the anion exchange material with an effective aqueous salt solution;

e) forming a third eluate by contacting the second eluate with a HPLC chromatographic material for a time sufficient for the molecule to bind to the material, and eluting the material from the hydroxylapatie HPLC; and

g) identifying fractions of said third eluate containing said molecule.

18. The method of claim 17, wherein the molecule is a membrane bound molecule, and the steps further comprises an initial step of isolating the molecule from the membrane.

19. The method of 17, wherein the purification is conducted in solutions containing one or more protease inhibitors at concentrations that effectively inhibit proteolysis of said molecule.

20. The method of claim 19, wherein the molecule is present in a solution comprising a reducing agent in an amount effective to preserve the activity of said molecule.

21. The method of claim 20, wherein the purification is conducted in solutions containing one or more metal ion chelators at concentrations that prevent substantial loss of activity of said molecule.

22. The method of claim 17, wherein the solution contains about 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, 0.5% NP-40, 0.5% deoxycholate, 1 μg/ml pepstatin and leupeptin.

23. The method of claim 17, wherein the cation exchange chromatographic material is S-Sepharose.

24. The method of claim 17, wherein the anion exchange chromatographic material is Mono Q HR 5/5 FPLC column.

25. The method of claim 17, wherein the HPLC of claim 15 comprises hydroxylapatite HPLC.

26. The method of claim 17, wherein the cation and anion exchange chromatographic materials, and HPLC, are S-Sepharose and Mono Q HR 5/5 FPLC column, and hydroxylapatite HPLC respectively.

27. A protein molecule, or fragments derived therefrom, that stimulates GTPase activity of rap, and comprises the partial amino acid sequences of: F(G)VSTKLPFT(W)DXA(Q)QL, IASNFL(P)AYIVVQAENPGTEPP(A)YK, and IASNFLSAYVVVQAEGGGPDGXLYKV, wherein X denotes an unknown residue and parenthesis indicates uncertainty in the enclosed residue.

28. An antibody to a substantially pure rap GAP.

29. The antibody of claim 28, wherein the antibody is a monoclonal antibody.

30. An antibody to a substantially pure protein molecule, or fragments of the protein molecule thereof, wherein the protein molecule comprises an apparent molecular weight of about 80,000 to 90,000 as assessed by SDS-PAGE under reducing conditions, that stimulates GTPase activity of rap.

31. A method for purifying rap GAP comprising binding an antibody of rap GAP to a matrix, contacting a solution containing rap GAP with the matrix to allow binding of the rap GAP to the rap GAP antibody to form a complex of antigen-antibody, and collecting rap GAP from the complex of antigen-antibody.

32. An antibody to a peptide sequence selected from the group consisting of F(G)VSTKLPFT(W)DXA(Q)QL, IASNFL(P)AYIVVQAENPGTEPP(A)YK, and IASNFLSAYVVVQAEGGGPDGXLYKV, wherein X denotes unknown residue and parenthesis indicates uncertainty in the enclosed residue.

33. The antibody of claim 32, wherein the antibody is monoclonal antibody.

34. A method for identifying cancer therapeutics, comprising the steps of:

a) forming a mixture by combining in a rap GAP assay compatible solution a compound suspected of being a cancer therapeutic, and rap GAP;

b) adding labelled rap·GTP to the mixture;

c) measuring the amount of GTP, GDP, or Pi in solution; and

d) relating the amount of GTP, GDP, or Pi in solution with a control sample prepared according to steps "a" to "c", wherein the control is free of the suspected cancer therapeutic.

35. Cancer therapeutic identified by the method of claim 34.

36. A method for identifying cancer therapeutics, comprising the steps of:

a) combining a labelled downstream substrate of rap·GAP-rap·GTP, with rap GAP, and a compound suspected of being a cancer therapeutic, in a solution compatible for the assay of a

downstream product of rap·GAP-rap·GTP interaction with the labelled downstream substrate;

b) measuring the amount of labelled downstream product; and

c) relating the amount of the labelled downstream product with a control sample prepared according to step "a", wherein the control is free of the suspected cancer therapeutic.

37. Cancer therapeutic identified by the method of claim 36.

38. A recombinant DNA sequence comprising a sequence substantially similar to the sequence selected from the group: HUB10A, HP3-12, and S, shown in Figure 10.

39. An isolated DNA which hybridizes to the recombinant sequence of claim 38.

40. A host cell transformed with the recombinant DNA sequence of claim 38.

41. A plasmid comprising the recombinant DNA sequence of claim 38.

42. Plasmid pAcRG9.

43. A recombinant DNA sequence encoding a polypeptide possessing rap GAP activity.

44. A recombinant DNA sequence encoding a polypeptide comprising an apparent molecular weight of about 80,000-90,000 as assessed by SDS-PAGE under reducing conditions, or a fragment derived therefrom, that stimulates GTPase activity of rap.

45. An isolated rap GAP protein having an amino acid sequence comprising the amino acid sequences selected from the group: HUB10A, HP3-12, and S, shown in Figure 10, or a functionally equivalent amino acid sequence thereof.

46. A method for producing an antibody to rap GAP protein or fragments thereof, the method comprising the steps of:

a) raising the antibody, in an animal, against a peptide comprising the rap GAP protein sequence or fragements thereof which retain antigenic activity; and

b) collecting the antibody.

FIG. 1A

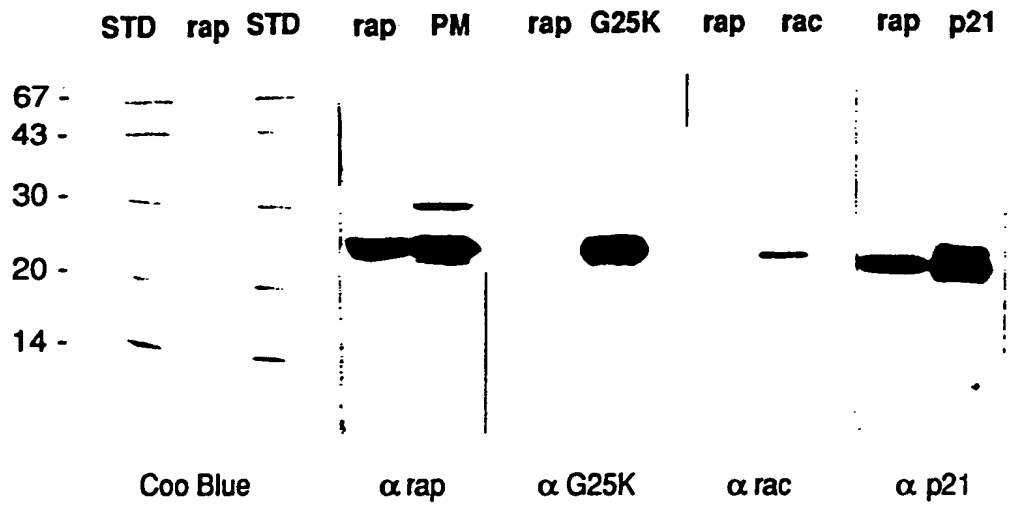

FIG. 1B

	1														16
rap 1B	M	R	E	Y	K	L	V	V	L	G	S	G	G	V	G	K
	‖	‖	‖	‖	‖	‖	‖	‖	‖	‖	‖	‖	‖	‖	‖	‖
NH_2-terminus of purified platelet protein	M	R	E	Y	K	L	V	V	L	G	S	G	G	V	G	K

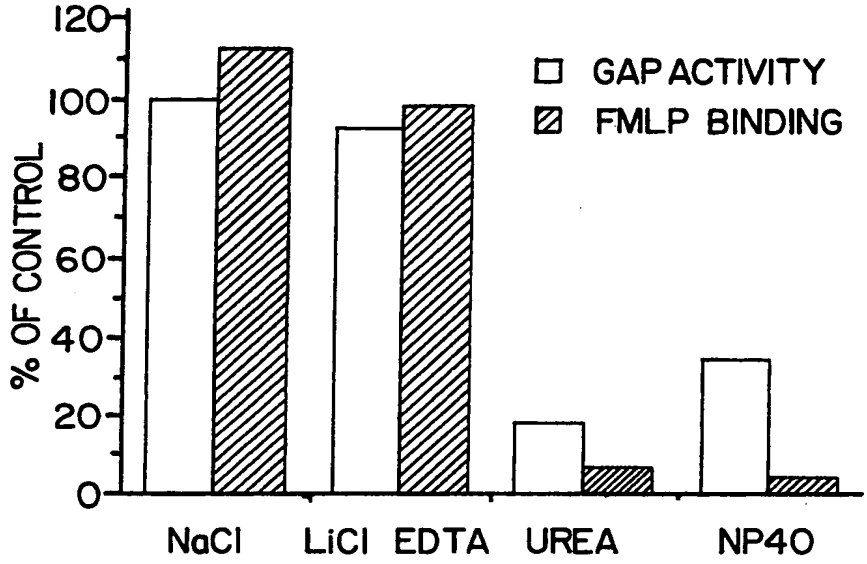

FIG. 2

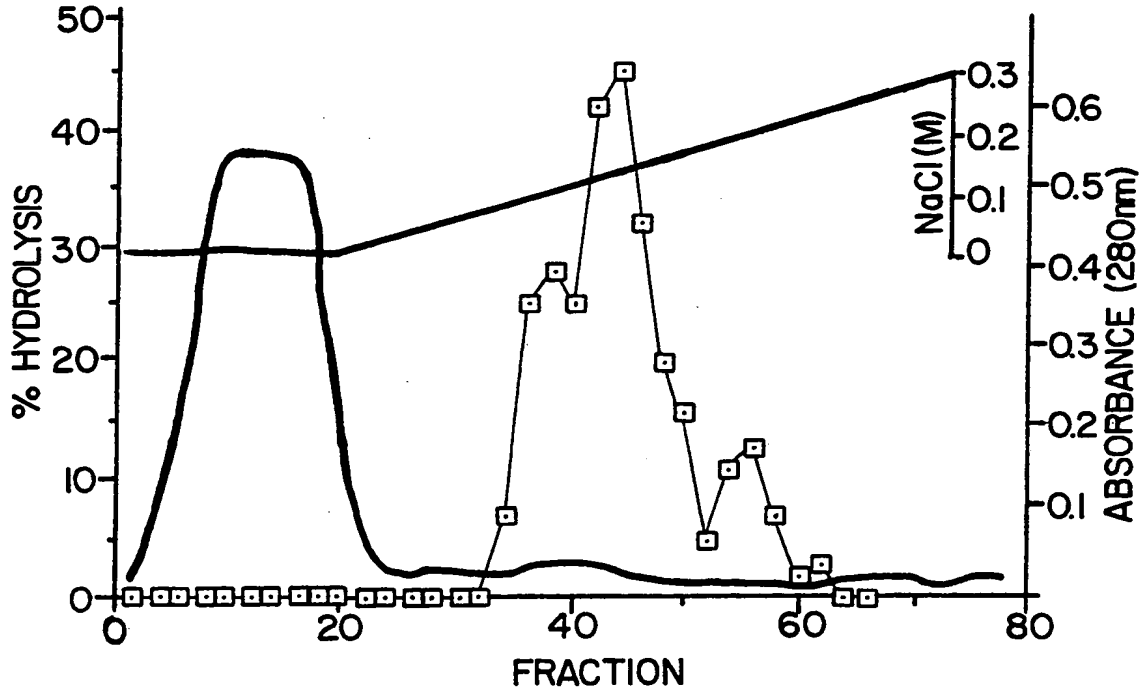

FIG. 5A

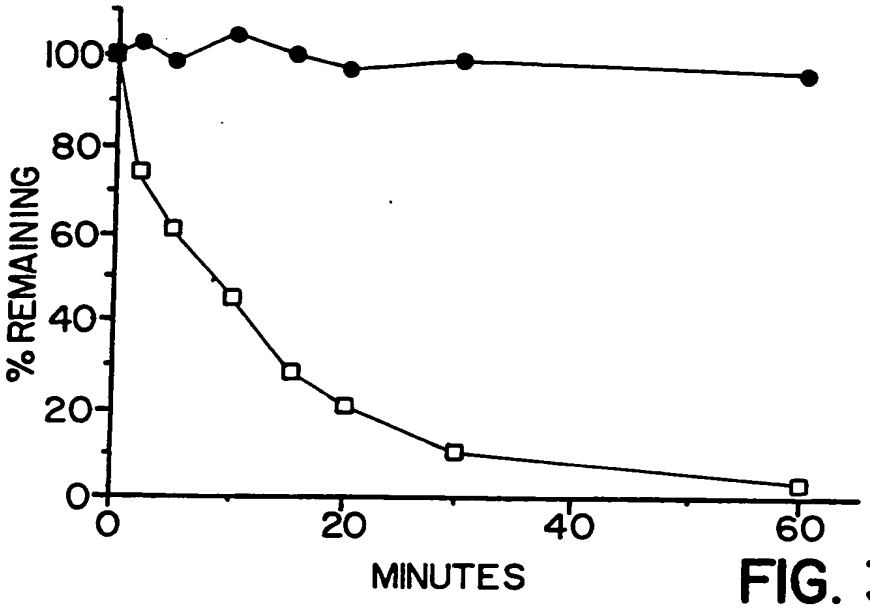

FIG. 3A

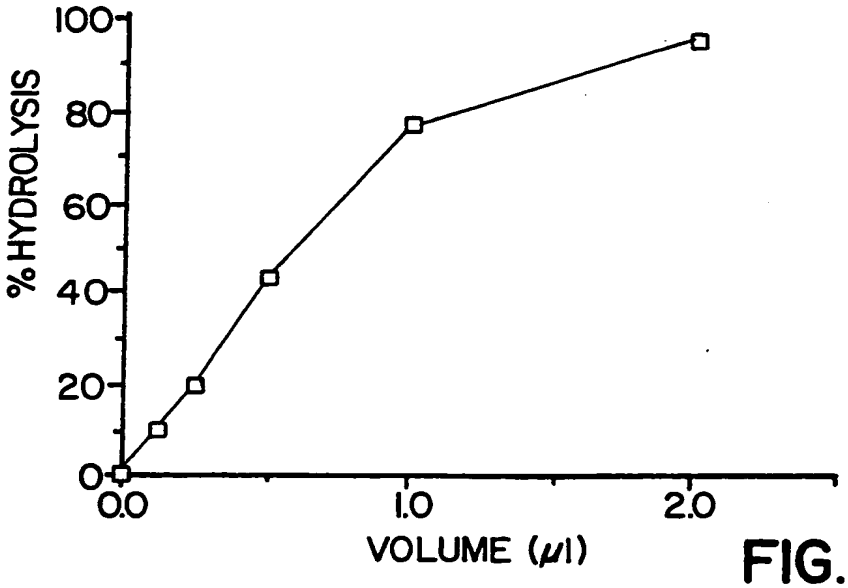

FIG. 3B

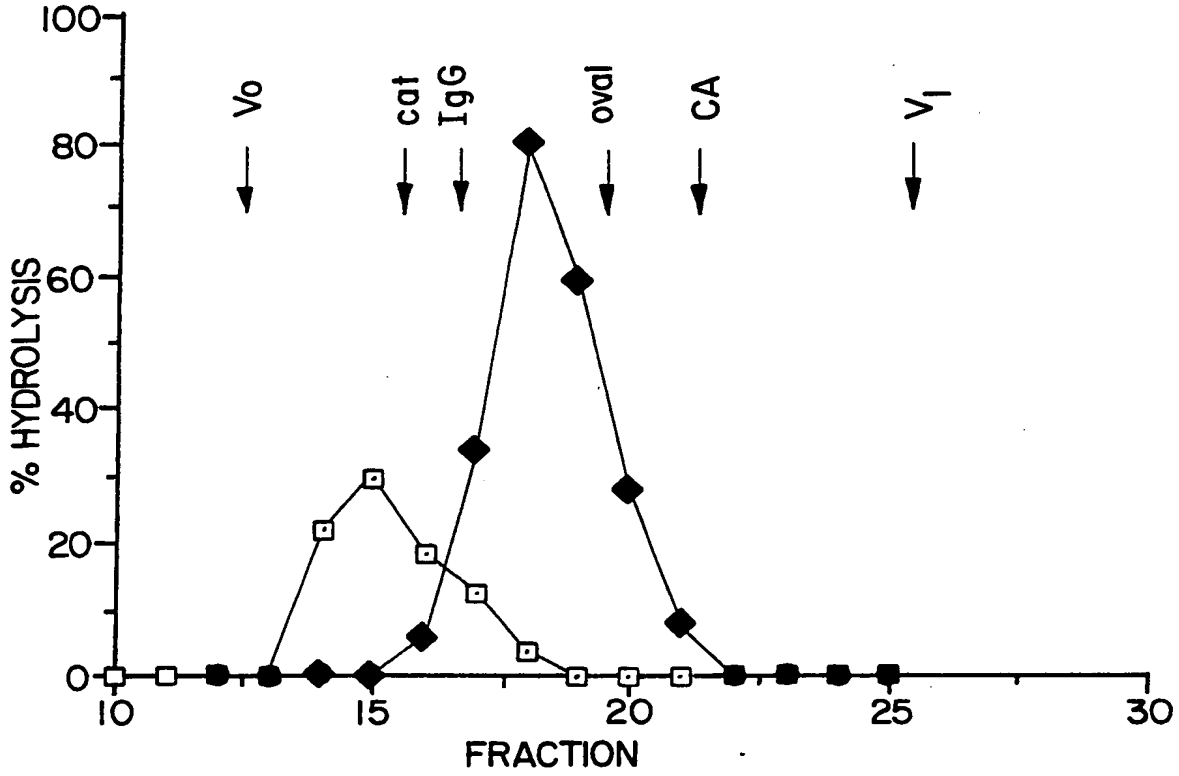

FIG. 4A

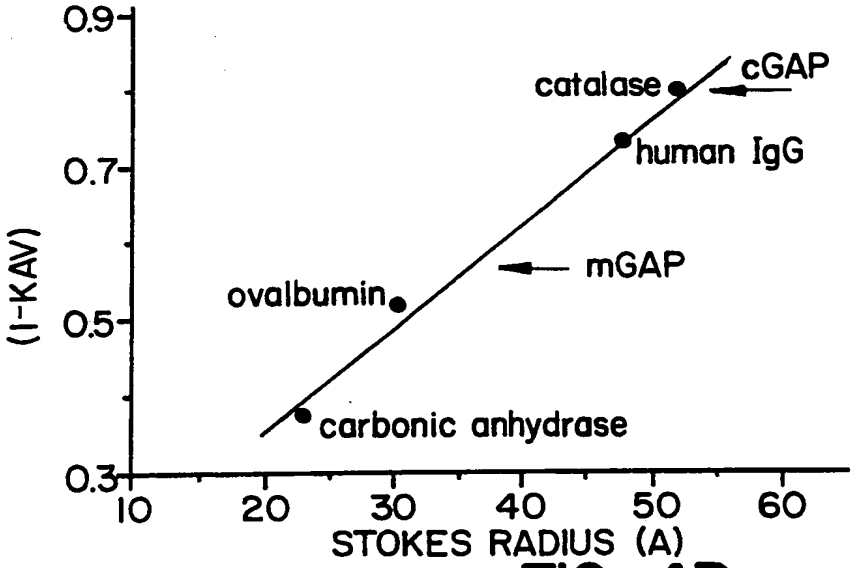

FIG. 4B

SUBSTITUTE SHEET

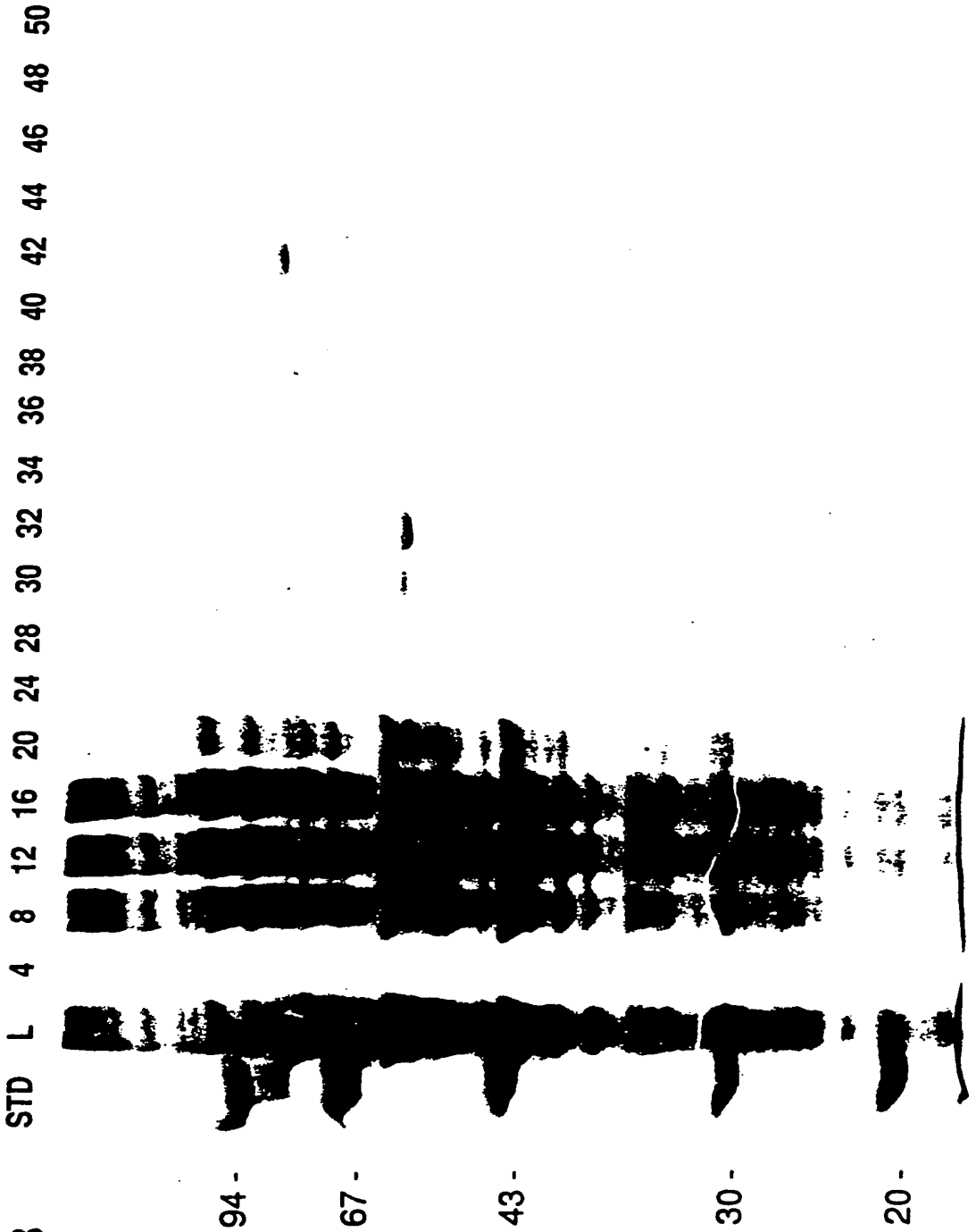

FIG. 5B

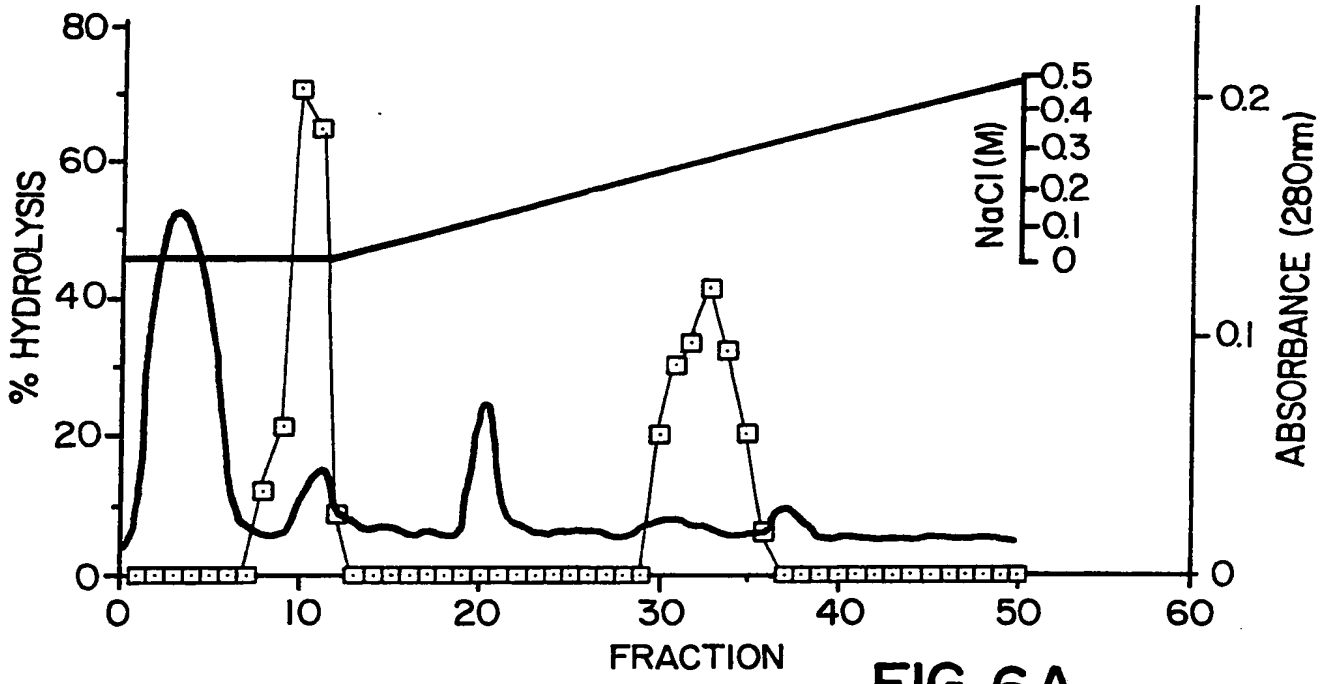

FIG. 6A

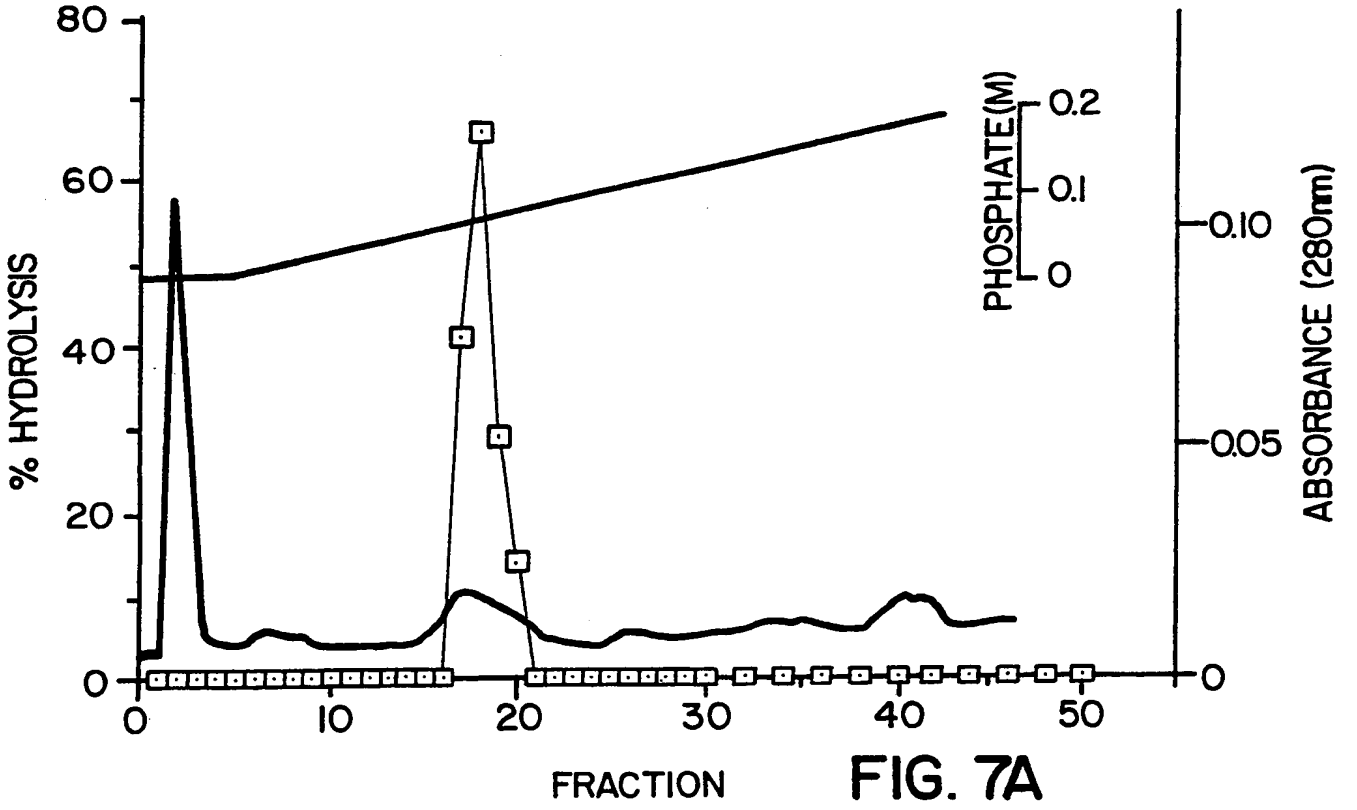

FIG. 7A

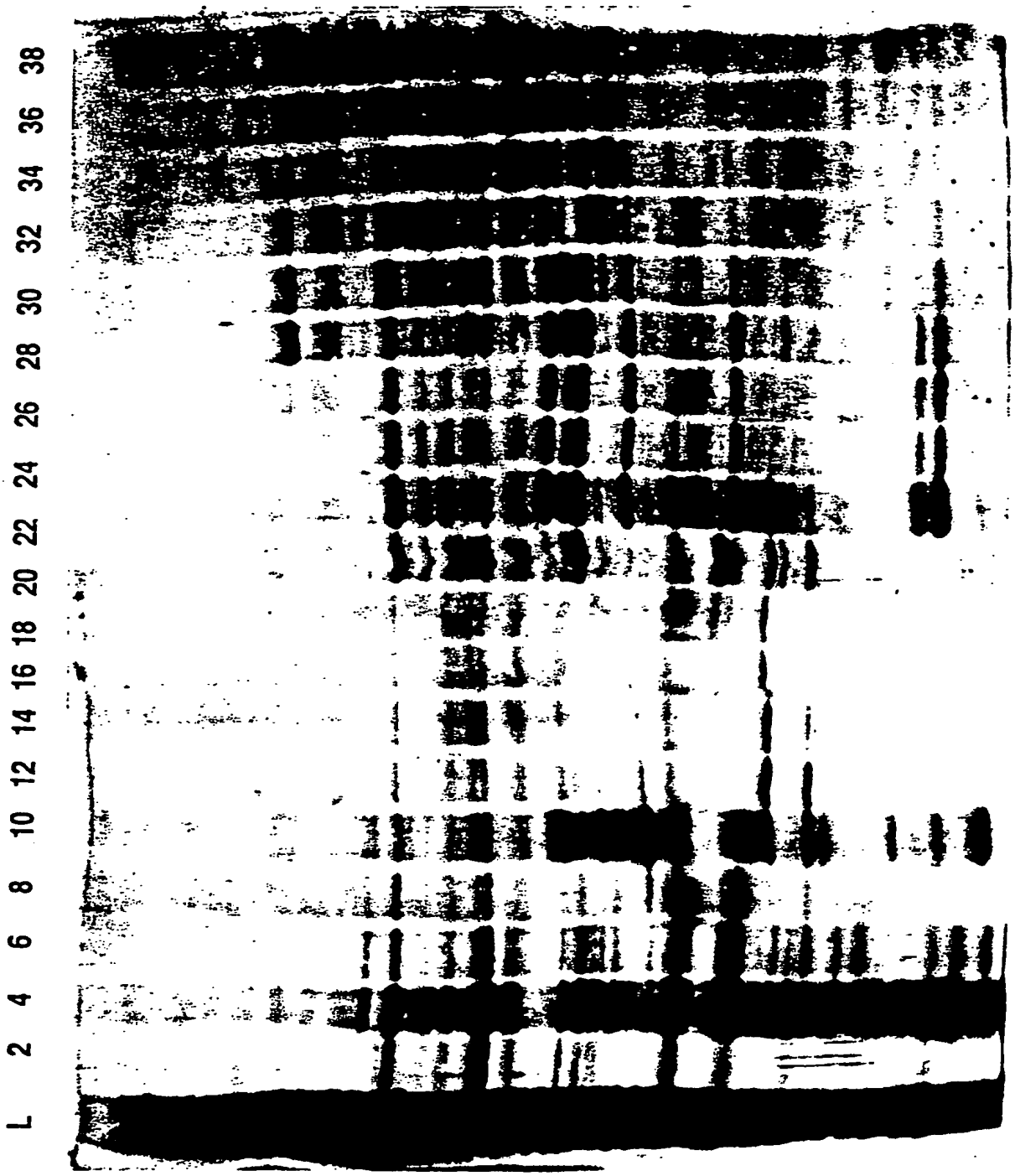

FIG. 6B

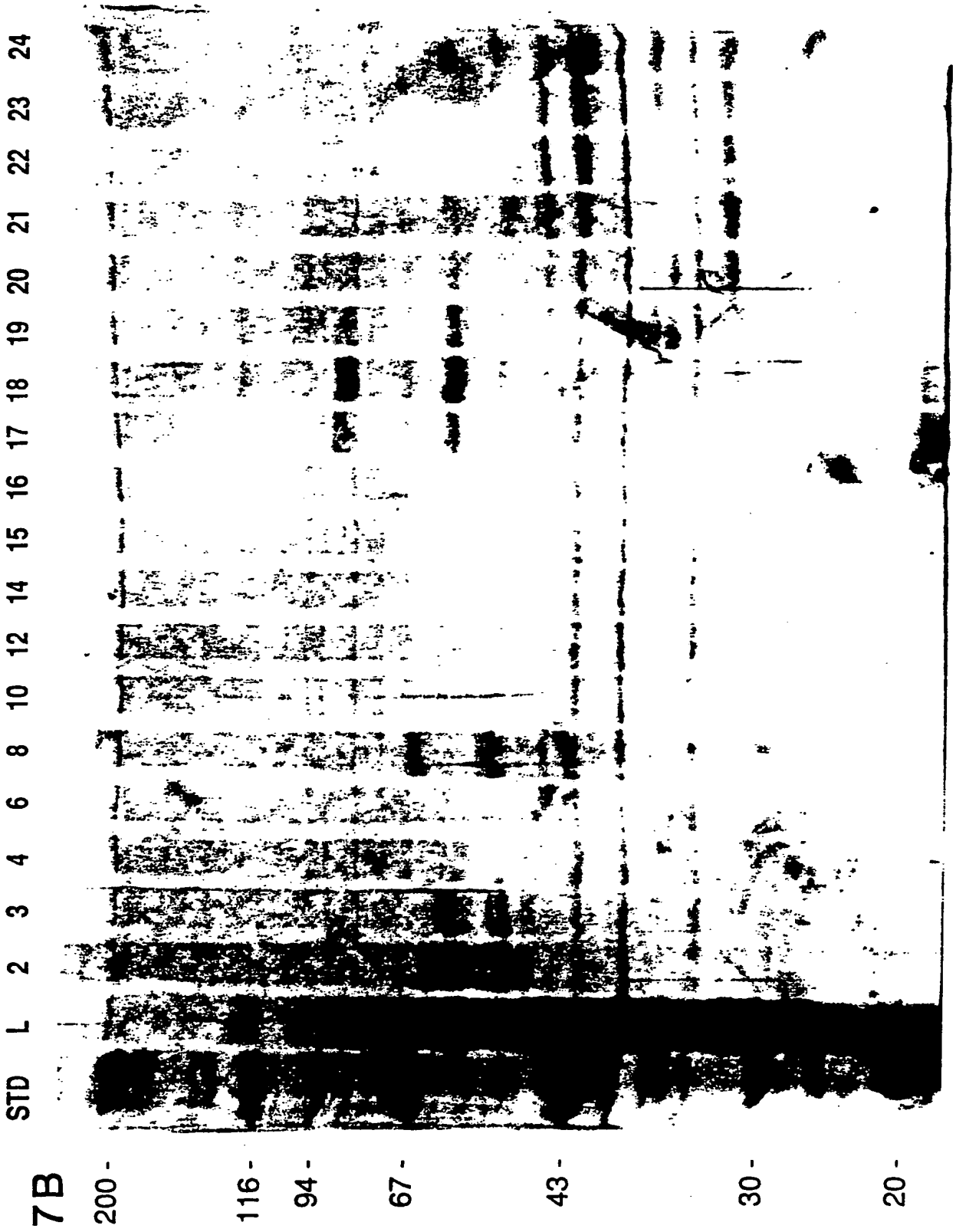

FIG. 7B

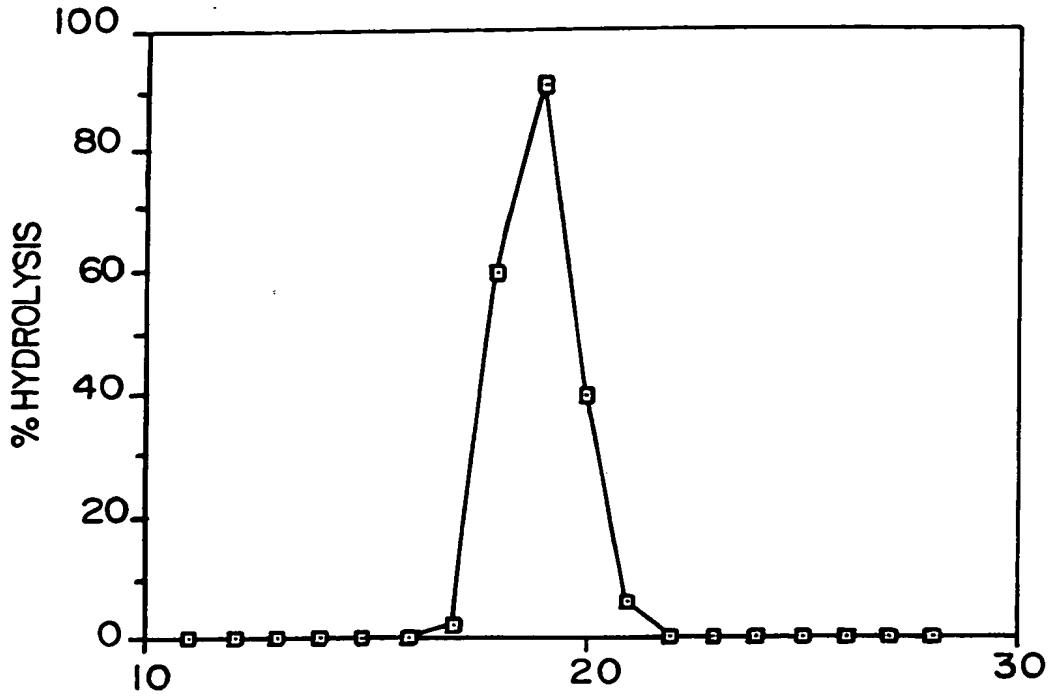

FIG. 8A

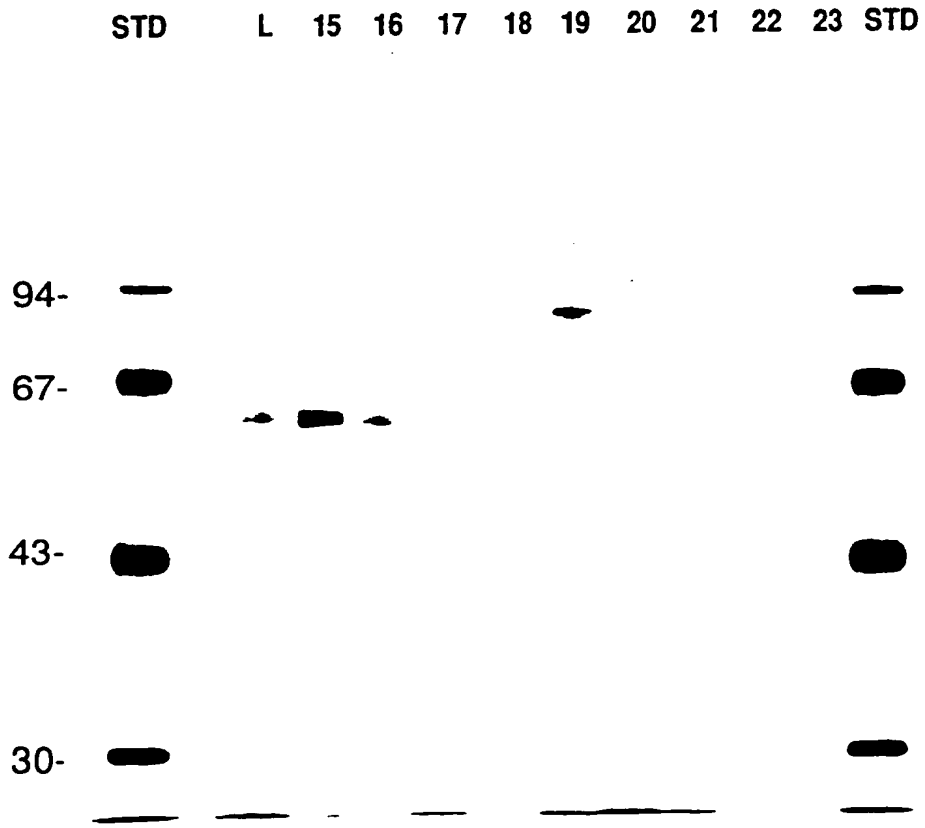

FIG. 8B

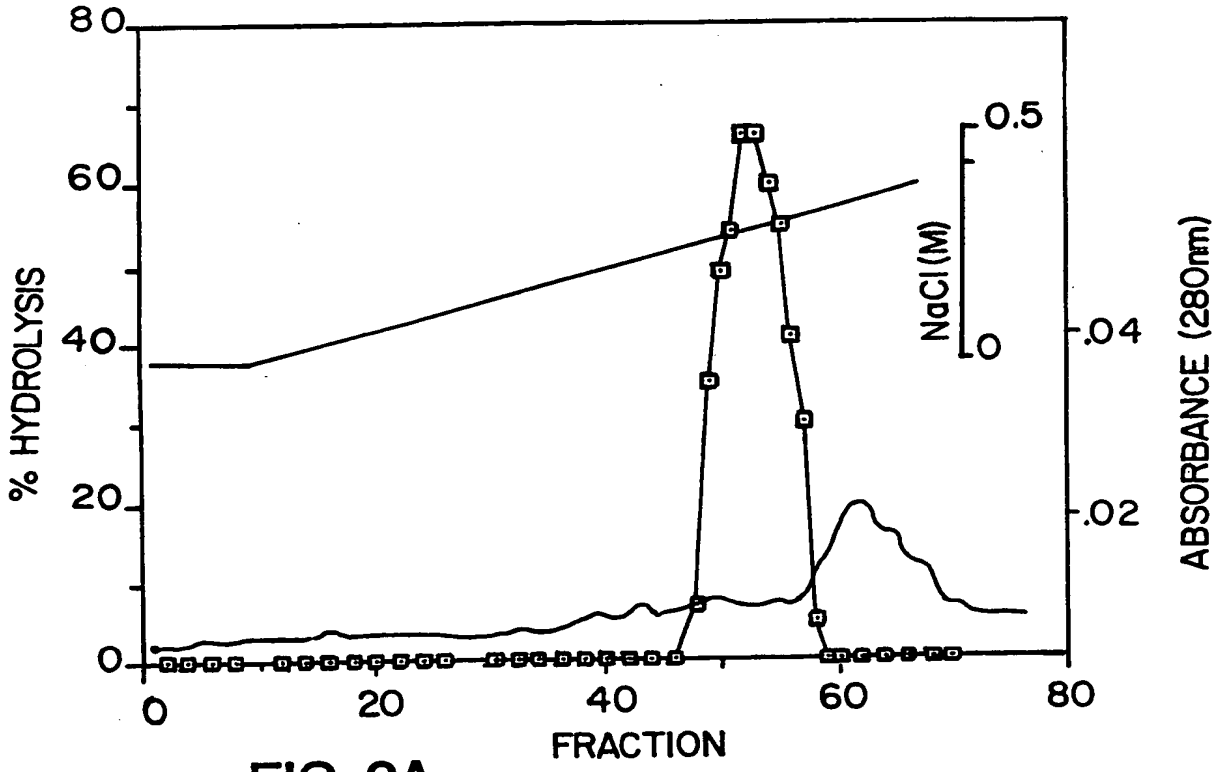

FIG. 9A

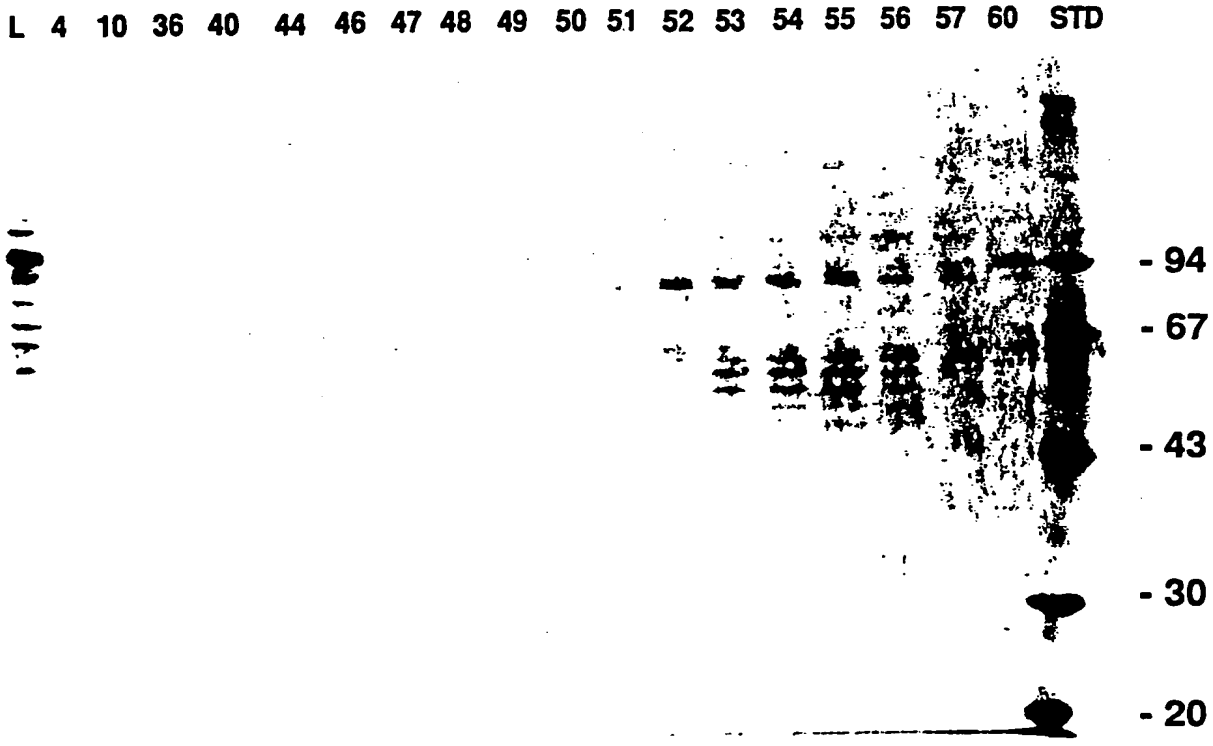

FIG. 9B

SUBSTITUTE SHEET

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                          M  I  E  K  M  Q  G  S  R  M  D  E  Q
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R  C  S  F  P  P  P  L  K  T  E  E  D  Y  I  P  Y  P  S  V
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H  E  V  L  G  R  E  G  P  F  P  L  I  L  L  P  Q  F  G  G
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GCCATAGGCATAGGAACCGTGGAAGAGTCACTGATTGTCCCTGGGAAGAGCCCCACGAGG
A  I  G  I  G  T  V  E  E  S  L  I  V  P  G  K  S  P  T  R
AAGAAGTCGGGCCCGTTCGGCTCCCGCCGCAGCAGCGCCATTGGCATCGAGAACATACAG
K  K  S  G  P  F  G  S  R  R  S  S  A  I  G  I  E  N  I  Q
```

FIG. 10A

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GAGGTGCAGGAGAAGAGGGAGAGCCCTCCGGCTGGTCAGAAGACCCCAGACAGCGGGCAC
E  V  Q  E  K  R  E  S  P  P  A  G  Q  K  T  P  D  S  G  H
GTCTCACAGGAGCCCAAGTCGGAGAACTCATCCACTCAGAGCTCCCCAGAGATGCCCACG
V  S  Q  E  P  K  S  E  N  S  S  T  Q  S  S  P  E  M  P  T
ACCAAGAACAGAGCGGAGACCGCAGCGCAGAGAGCAGAGGCGCTCAAGGACTTCTCCCGC
T  K  N  R  A  E  T  A  A  Q  R  A  E  A  L  K  D  F  S  R
TCCTCGTCCAGTGCCAGCAGCTTCGCCAGCGTGGTGGAGGAGACGGAGGGTGTGGACGGA
S  S  S  S  A  S  S  F  A  S  V  V  E  E  T  E  G  V  D  G
GAGGACACAGGCCTGGAGAGCGTGTCATCCTCAGGAACACCCCACAAGCGGGACTCCTTC
E  D  T  G  L  E  S  V  S  S  S  G  T  P  H  K  R  D  S  F
ATCTATAGCACGTGGCTGGAGGACAGTGTCAGCACCACTAGTGGGGGCAGCTCCCCAGGC
I  Y  S  T  W  L  E  D  S  V  S  T  T  S  G  G  S  S  P  G
CCCTCTCGATCACCCCACCCAGACGCCGGCAAGTTGGGGGACCCTGCGTGTCCCGAGATC
P  S  R  S  P  H  P  D  A  G  K  L  G  D  P  A  C  P  E  I
AAGATCCAGCTGGAAGCATCTGAGCAGCACATGCCCCAGCTGGGCTGTTAGCCGGGCCAC
K  I  Q  L  E  A  S  E  Q  H  M  P  Q  L  G  C  *
CCCCTCTGAAGGTGAAACTGAGCAGATGAGGCCACAGAAGCACAAGGGGAAGGTGCCGTG
TCAAGCCCAGGCAGACGAGACCTCTGCCCTGAAGACCAACACCAGCCCGTGGGCTGCCCC
CTGCCTCCCCACCCTCCCCATGGCCCACCCATCTGGGCTGTCTCTGCAGGGCAGAGCCGT
CCAGACCTGGGATCAGGGAAGCTGCTGGCATCGTCCCCACCCCCAGCCTGGGGGTCTGGG
CTGGGGCAGGGATTGCTCAGTGGAAGCAGGACTGGGGGTCTGGCTTGCCCCCTCCCTGGG
CCTCCATCACCCCTGAGCATCCCTCTGGACTCAGAGGGAACAAGGTGGGAGAGAGAGTTT
GAGACAGCTCCGTGTGGAGAGCTTAGCCCCTGGAGGCAGCACAAGGAGGATGTGATATGT
GGGGGAGTGAGCACTGGGTTGGGAGCCGGGTCCTGGTTTCCAATTTGGGTTCTGCTGTGT
GACTCTGGGCAAGTCACTCTCCCTCTCTGGGCATGTCTGCTACAAATGGACAAGATTATT
TCAGAGGTCACTGAAGACTGTGATTACATGCACCTGCCTTAGAAGGTAGGATTTTCTTCC
CAGGGACCTCCTATCACCCTACCCTGCTTCTTGAGGTCCCTGGAGCCCCAGGTGGGCTGA
GGGGCAGGGAGCCGGCTGTGCCCAGTATGCCTCCTGGACCCTCCAGTTCTGCCACAGGTC
TGCCGATGCCCTGTCCACTGCCTACACATGACAGACAAGTAACCCCCTCATGGGGGATGG
GGACCTACCTGGCTCCTCAGCCAGCACCCAGCTTAACCCCTGCCATCCCATGCTGGGCCC
TCCAGGCCAAGAGTCTCAGCTGGCCGAGAGTCCAGGCCTTGCCTCCCCGACCGCCATGGA
GGGGGCAGCCCGGCACAGCTGCTGGGAGCCCTTGTGTGTCTGGTCACACTTTTTAGGCGT
CACGCCAAAGGCCAGCCTCCTGGCCCCAATACCCATTTTGGAAGCCCCTGTGGCCGTGTG
GATGTCGGTAACAGTTGTATAAAATAAATTCTATTATCGCTATTGTAAAAAAAAAAAAAA
AAAAAAAAAAAAAAA
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[ ] HP3-12
( ) Sequence S
Entire Sequence HUB10A

FIG. 10B

FIG. 11A

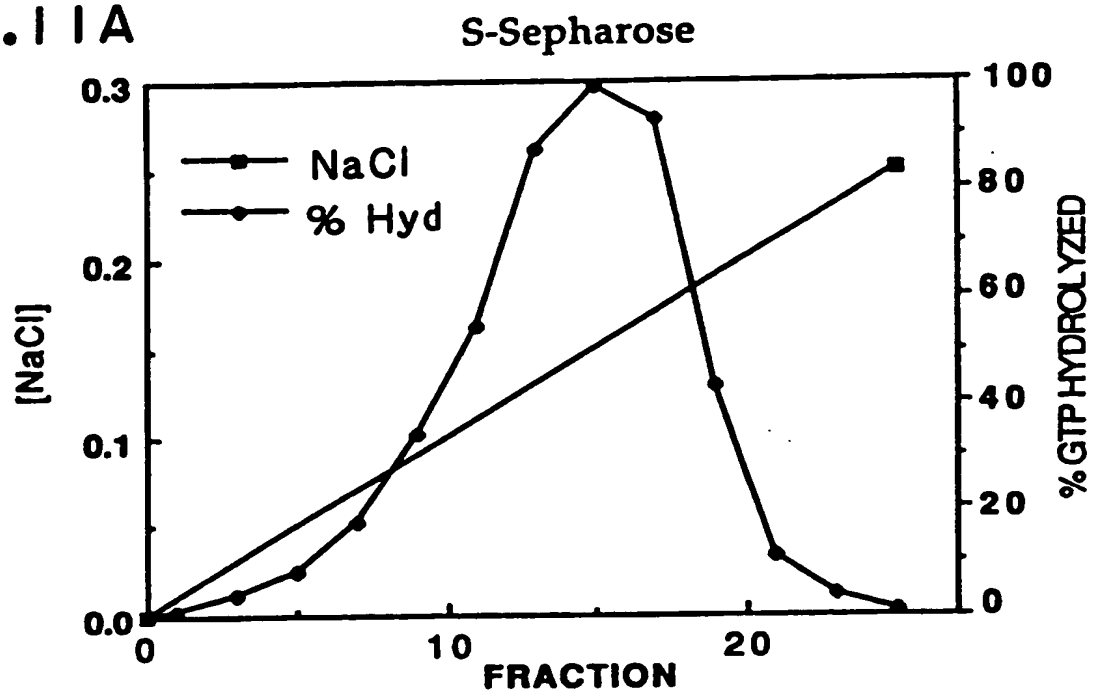

FIG. 11B

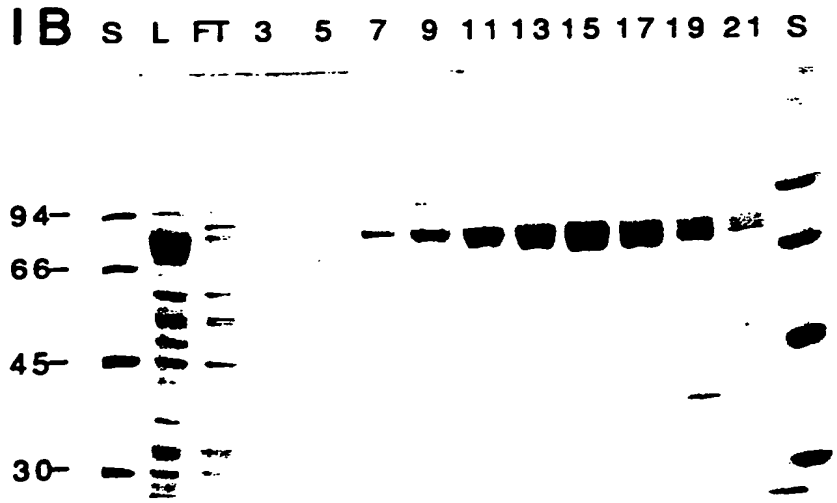

FIG. 12